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CHEMICAL BLISTERING: CELLULAR AND MACROMOLECULAR COMPONENTS

ANNUAL REPORT

I. A. BERNSTEIN

JANUARY 30, 1987

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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The overall objective of this investigation is to elucidate in culture the molecular mechanisms by which bis-(beta-chloroethyl)sulfide (BCES) exerts its vesicant action when applied topically to human skin.

The technical objectives of the project are to establish morphological, cytochemical and/or biochemical indicators of epidermal mustard toxicity and to examine and evaluate the relevance of selected parameters to vesication.

Three biological systems used in this study of the molecular and cellular effects of exposure to BCES are a) the stratified terminally differentiating primary culture of cutaneous keratinocytes derived from human or rat skin grown on a collagen gel or nylon membrane as substratum positioned at the air-medium interface; b)the submerged monolayer of proliferating and early

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19. Abstract (continued)

differentiated keratinocytes grown on the plastic surface of the Petri dish, and c) purified populations of basal cells, which are obtained by sedimentation in a density gradient and are used as innocula for cell culture.

Submerged monolayer cultures exposed to as little as 0.1 uM BCES exhibited single strand breaks in the DNA which were completely repaired within the following 22 hours. Although all cells survived the treatment and remained in the culture, about 20% of the cells failed to incorporate [^{3}H]thymidine in the subsequent 24 hours, a period of synchronized DNA replication in the untreated culture. It appears that the repair of single strand breaks was not coincident with complete recovery of cellular damage. BCES-mediated abnormalities in cell surface carbohydrates, in keratin maturation, and in insulin-binding require higher levels of exposure than does the effect on DNA. The same is true for ultrastructural changes.

Basal cells are more susceptible to DNA damage than are differentiated cells, which could explain the observation that in a lifted culture, necrosis appears first in the basal cell layer. This morphological change appears to coincide with the structural changes associated with vesication in the human skin in vivo.

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FOREWORD

The source of animal tissue for primary cultures described in this report was neonatal rats derived from the CFN strain by random mating and reared in the School of Public Health's animal facility. This facility is under the supervision of the University Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Accreditation Association for Laboratory Animal Care (AAALAC).

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 86-23, Revised 1985].

The source of human tissue was foreskin obtained at routine circumcisions done at Women's Hospital, The University of Michigan, and provided without identification of the donor. The form utilized to obtain"informed consent" was the one in use by the hospital for routine circumcision. Signature on this form allows experimental use of tissues.

The use of this tissue for the present project has been approved by a University Human Subjects Review Committee and, for the protection of human subjects, the investigators have adhered to applicable policies of Federal Law 45 CFR 46.

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NARRATIVE REPORT OF PROJECT PROGRESS

A. Statement of the Problem under study

The overall objective of this investigation is to elucidate the molecular mechanism by which bis-(beta-chloroethyl)sulfide (BCES) exerts its vesicant action when applied topically to human skin. The theoretical and technical advances which have occurred in cutaneous biology over the past several decades encourage the view that this objective can be achieved. It appears likely that the most productive application of the new knowledge would be in experiments involving cultures of epidermal keratinocytes. Studies in vitro should allow better controlled experiments and yield more reproducible data than is true in vivo.

The technical objectives of this project are to establish morphological, cytochemical and/or biochemical indicators of epidermal mustard toxicity and to examine and evaluate the relevance of selected parameters to vesication. This information should aid in elucidating the molecular etiology of the cutaneous injury from exposure to BCES and in providing a rational basis for proposing and testing prophylactic and therapeutic measures.

The specific aims which guide the study are as follows.

1. To identify the most sensitive biochemical and/or morphological BCES-mediated lesion(s) in lifted cultures of cutaneous keratinocytes by

a. establishing dose response curves for damage to and recovery of the following macromolecular and cellular parameters in basal and differentiated cells of submerged monolayers exposed to the agent:

1) deoxyribonucleic acid (DNA)

2) mitosis

3) differentiation

4) cellular ultrastructure.

b. comparing the dose response curves to identify the earliest and most sensitive toxic responses in BCES-treated keratinocytes and to determine the relative susceptibility of basal and differentiated cells to the mustard.

c. establishing that lifted cultures, topically exposed to BCES under reproducible conditions of application and penetration of the agent, exhibit the same abnormalities found in submerged monolayers.

2. To ascertain the relevance of the abnormality seen at the lowest dose of BCES in lifted cultures to the cellular necrosis which is requisite for vesication by

a. evaluating the early toxic response in regard to the following macromolecular and cellular activities in the monolayer:

1) disappearance of the lesion from DNA

2) mitotic rate

3) differentiation.

b. determining the ability of the cells in the exposed monolayer to develop biochemically and morphologically normal lifted cultures.

B. Literature Background

1. Anatomy of blistering

Application of BCES to human skin results in an initial erythema followed by blistering. Stoughton (1971) has noted that in vesication "fluid accumulation is almost always secondary to fundamental damage to the cellular structures" and has defined a blister as "an abnormal accumulation of fluid, completely replacing the pre-existing tissue structure, capped by a part or all of the epidermis." The blister seen after exposure to BCES fits this description. The progression to ultimate blistering from this agent proceeds irrevocably unless the action of the chemical is neutralized within the first several minutes of exposure. Warthin and Weller (1919) and Sinclair (1949) noted that the process initially involves destruction of the basal and lower spinous layers of the epidermis. Presumably, the cellular destruction that produces a cavity in the tissue precedes the actual fluid accumulation. At later stages in the destructive process, the necrosis may spread to the upper spinous and granular layers as well as into the dermis. Blistering is rare in animals and appears to be a reaction primarily seen in man, in whom it may arise as a component of various cutaneous diseases and as a result of exposure to some biological (e.g., Herpes virus), physical (e.g., ultraviolet radiation) or chemical (e.g., mustards) stressors. The blister may develop at different levels in the skin depending upon the etiologic agent involved.

2. Biochemistry of blistering

Since different vesicants produce blisters at different levels in the tissue, it would not be unexpected if the biochemistry involved were different depending on the location of the blister.

Exposure to a mustard <u>in vivo</u> will cause separation at the dermal-epidermal junction; i.e., the blister appears at that level in the tissue. From a description of the time course of dermatopathologic development and the time postexposure within which the process can be reversed, it is clear

that the molecular course of the pathologic process is set within the first 3 min of exposure in vivo. Warthin and Weller (1919) found that erythema, inflammation and vesication were not reversed by therapy (i.e. application of chlorinated lime) initiated after 3 min of exposure to BCES.

The molecular mechanism of BCES's effect is unknown, but substances in this class are powerful alkylating agents of DNA. Alkylated DNA could lead to inhibition (or at least delay) of replication; to generalized breakdown of damaged DNA resulting in cell death (cf, Wheeler, 1962); and to low-fidelity repair, resulting in mutations with consequent disruption of normal metabolic function (Kirner, 1946; Wheeler, 1962). Mustards can also alkylate RNA with consequent interference in the translation of genetic information and in protein synthesis (Ross, 1962), resulting in metabolic disruption (Wheeler, 1962). BCES, being a bifunctional mustard, can also cross-link DNA to DNA, RNA or protein. The most important molecular target appears to be the DNA (Fox and Scott, 1980). BCES alkylates and forms cross-links at the purine bases. Alkylation of the phosphate groups in DNA can also occur. On the basis that damaged proteins can be replaced whereas damage to DNA may result in irrevocable change, the DNA would seem to be the most important target for toxicity from exposure to BCES.

Exposure of isolated skin to a vesicant for as little as 5 min can result in the inhibition of glycolysis and respiration (Barron, et al., 1948). Glycolysis is inhibited by a lower concentration of toxicant than is respiration. In the case of exposure to mustards, this effect seems to be a consequence of a reduced level of pyridine nucleotides in the cell (Holzer and Kroger, 1958; Frazer, 1960). Recent evidence suggests that BCES causes a lowered level of pyridine nucleotides by virtue of the stimulation of poly ADP-ribose polymerase (Gross, et al., 1983).

3. Repair of alkylated DNA

In bacteria, the ability to excise BCES-alkylated products from DNA is associated with increased resistance to the mustard (Lawley and Brookes, 1968). Apparently both mono- and bifunctional adducts can be removed from DNA, since comparable amounts of induced repair synthesis have been observed in HeLa cells exposed to BCES and the analogous half-mustard; the compounds are equally toxic (Roberts, et al, 1971). However, there appears to be disagreement as to the relative rates at which repair of the two types of lesion occur (Reid and Walker, 1969; Roberts, et al, 1971). Given the mechanisms probably involved in the two repair processes, it seems unlikely that removal of the cross-links could occur as quickly as removal of monofunctional adducts (cf, Fox and Scott, 1980). Intuitively, it appears more likely that the cross-link would be removed one arm at a time rather than both arms simultaneously since the latter process would insert a double strand break which could be lethal. Of course, this analysis assumes that the mechanism of repair is not simple base replacement. Detailed information on the molecular mechanisms by which BCES-induced lesions in DNA are repaired does not seem to be available.

4. Cultivation of keratinocytes

The two main types of cells in the skin are fibroblasts (dermal) and keratinocytes (epidermal). In the epidermis, only the keratinocytes existing at the dermal - epidermal junction can double their DNA and undergo mitosis although all nucleated keratinocytes can repair their DNA (Karasek and Moore, 1970; Vaughan and Bernstein, 1971). Fibroblasts are easily cultivated using basal medium supplemented with serum (Earle, 1958). Cultivation of keratinocytes requires more stringent conditions. Several systems are available for cultivating keratinocytes. The best involve use of a substratum of collagen (Karasak and Charleton, 1971; Freeman, at al., 1976) or a feeder layer of irradiated fibroblasts (Rheinwald and Green, 1975). Conditioned medium is also useful (Ham, 1982) but not necessary (Peehl and Ham, 1980; Eisinger, et al, 1980). Good growth can be obtained by supplementing the medium with growth factors such as epidermal growth factor (Cohen and Savage, 1974); Rheinwald and Green, 1977) and hormones (Hayashi, et al, 1978). Vaughan, et al. (1981) reported successful cultivation, passage and increased plating efficiency of murine keratinocytes after supplementing basal medium with hydrocortisone and insulin.

Most of the cultures of keratinocytes mentioned above form monolayers, with some multilayering and production of cornified layers. However, they do not reproduce structural characteristics typical of their counterparts in situ. There have been previous reports of stratification of rabbit, human and rat keratinocytes with some cellular characteristics similar to intact epidermis (Karasek and Moore, 1970; Vaughan and Bernstein, 1971; Kitano, 1979). Lillie, et al. (1980) cultured a line of rat lingual epithelial cells at the air-liquid interface by lifting collagen-supported cultures on organ culture grids. This resulted in stratification and terminal differentiation with organellar components similar to those of the parent tissue. Modification of this technique led to the development of protocols for the successful production of an "epidermis" from primary isolates of cutaneous keratinocytes (Bernstein, et al. 1985; Vaughan, et al. 1986; Bernstam, et al. 1986).

C. Experimental Rationale for the Investigation

A tissue culture of cutaneous keratinocytes provides a biological system in which the direct interaction of BCES with molecular and cellular elements can be studied without the systemic influences which secondarily affect the toxic manifestations. Since the culture has the morphological and biochemical characteristics of the epidermis in situ, the mode of human exposure, topical application, can be mimicked experimentally and the results can be credible in terms of the situation in vivo. Furthermore, by the use of human keratinocytes, the conditions of the study model the "human condition" without necessitating trauma to a human volunteer.

Molecular parameters of toxicity are tested in the submerged monolayer culture to establish a range of effective exposures to BCES. The intent was to determine which indicators of toxicity appear at the lowest exposure and then to determine whether these parameters are indeed relevant to toxicity as observed in stratified cultures after topical application of BCES at

dosages equivalent to those which produce a toxic response in vivo.

D. Progress Report (1 January 1986 - 31 December 1986)

The specific aims of the original contract proposal and progress toward their achievement to date are as follows:

1. To identify the most sensitive biochemical and morphological BCES lesion(s) in lifted cultures of cutaneous keratinocytes

a. By establishing dose response curves for damage to and recovery of the following macromolecular and cellular parameters in basal and differentiated cells of submerged monolayers exposed to the agent:

1). Deoxyribonucleic acid.

The nucleoid sedimentation assay (Cook, et al. 1976; Romagna, et al. 1985) provides a very sensitive technique for determining the presence of single strand breaks in DNA. Nucleoids consist of supercoiled DNA associated with a small amount of non-histone protein obtained from cells by gentle lysis at neutral pH in the presence of a non-ionic detergent. The assay involves sedimentation of the nucleoids in a 15 to 30 percent linear gradient of sucrose containing 2M NaCl, 10mM EDTA, 10mM Tris buffer (pH 8) and a dye that interacts with DNA, causing the nucleoid band to fluoresce when illuminated with light at 366 nm. Single strand breaks cause the supercoiled DNA to relax. Nucleoids made from relaxed DNA sediment more slowly in the gradient than do nucleoids made from supercoiled DNA; the decrease in the rate of sedimentation is proportional to the amount of damage (i.e., single strand breakage) in the DNA.

To carry out the assay, keratinocytes, separated from the culture dish by exposure to a solution of dilute trypsin-EDTA, are collected by centrifugation at 50 xg at 4° C and lysed by suspension in a mixture of 2M NaCl, 11.7 mM EDTA, 10mM dithiothreitol, 11.7 mM Tris and 0.6% Triton X-100 at pH 8. The lysate is placed on top of the sucrose gradient in a centrifuge tube and the resulting nucleoids are sedimented at 25,000 xg for 20 to 25 min at 20° C. This is a very sensitive assay and can be used to measure only small amounts of damage to the nucleic acid.

Using this technique, it has been possible to establish a dose response relationship over a range of exposures from 0.1 to 15 uM BCES for 1 hour (Figure 1). The assays were carried out immediately after 1 hour exposure. A dose of 10 uM BCES produced a maximal response in the assay, although higher doses undoubtedly caused further damage which was undetectable by the nucleoid sedimentation technique.

To determine whether exposed cultures could recover after exposures in this concentration range, the exposed cultures were washed to remove free BCES and incubation was continued in the usual growth medium. At 22 hours post-exposure, the cultures were analyzed by nucleoid sedimentation. As noted in Figure 2, complete recovery was achieved, as

determined by this technique, if the exposure had not exceeded 5 uM.

In spite of this apparent recovery from the "single-strand break" lesion by 22 hours post-exposure, the cells exposed to only 0.1 uM BCES were not normal. At 24 hours after exposure to 0.1 uM BCES, the incorporation of [^{3}H]thymidine into nuclear DNA after a one-hour pulse of the tracer was only 80 percent of normal (Figure 3). After an exposure to 1 uM BCES, the incorporation of tracer was about 60 percent of normal; the culture also had lost about 20 percent of cells based upon the decrease in the total amount of DNA relative to that in the control culture. Figure 4 shows the amount of DNA

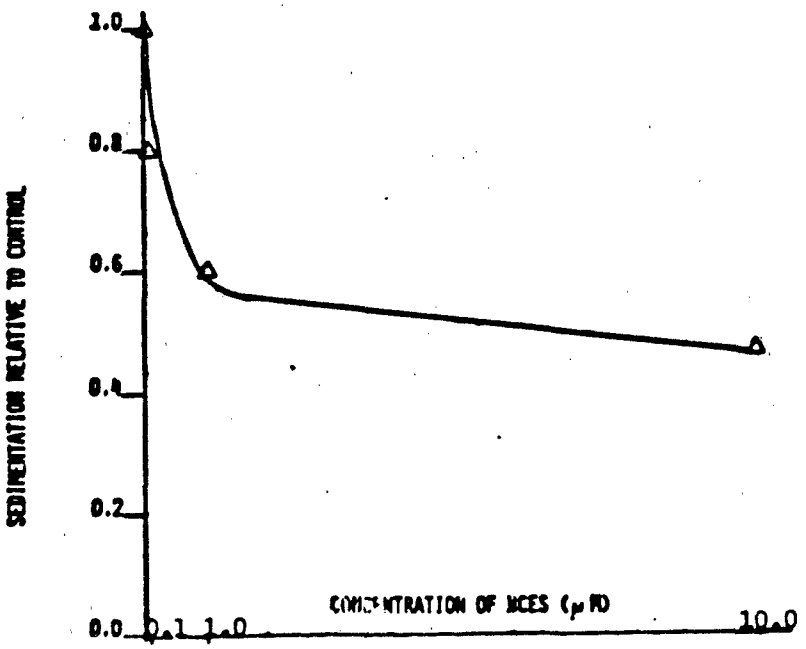


Figure 1. Sedimentation rate of nucleoids derived from monolayers of keratinocytes immediately after exposure for 1 hour to 0.1 to 10 uM BCES. For technical details see the TEXT.

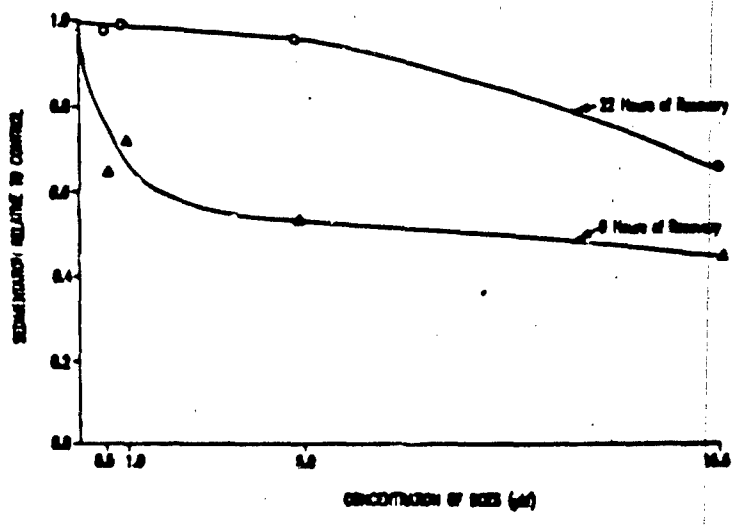


Figure 2. Sedimentation rate of nucleoids derived from monolayers of keratinocytes exposed for 1 hour to 0.1 to 10 uM BCES, washed and incubated for 22 hours in fresh growth medium. Cultures grown in low-calcium medium.

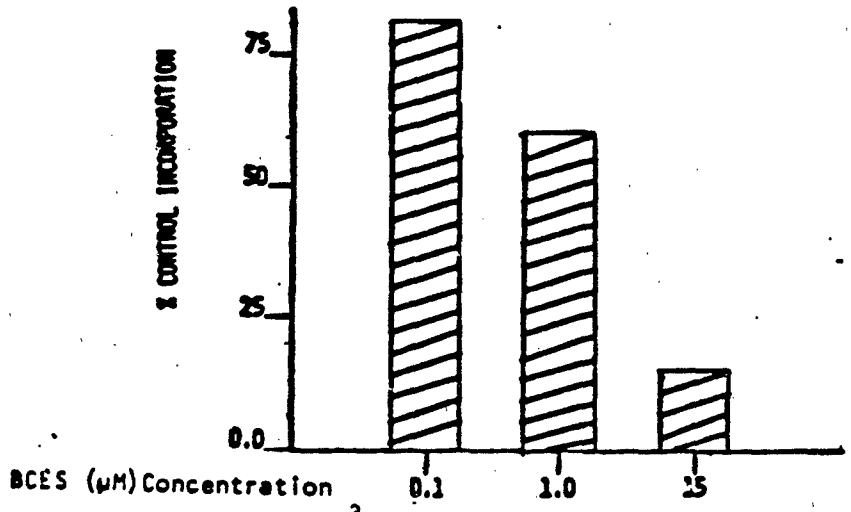


Figure 3. Incorporation of [^{3}H]thymidine in monolayer cultures of rat keratinocytes 24 hours after exposure for 1 hour to different concentrations of BCES. Cultures were exposed to 7% trichloroacetic acid at room temperature for 10 minutes and washed with potassium acetate (9.8 g/l) in absolute ethanol followed by absolute ethanol. The residual insoluble material was extracted with 0.1 N NaOH at 60° C for 15 minutes and aliquots of the extracts were counted in a liquid scintillation spectrometer.

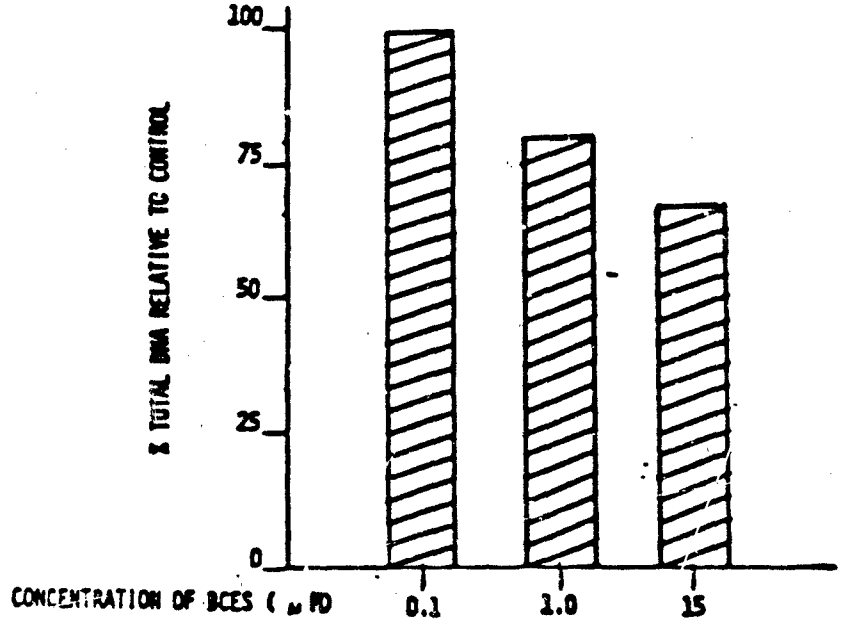


Figure 4. Content of DNA in monolayer cultures of rat keratinocytes 24 hours after exposure for 1 hour to different concentrations of BCES. DNA was determined by the method of Setaro and Morley (1976).

remaining in the culture 24 hours after exposure to different concentrations of BCES. Furthermore, of the cells remaining in the culture after exposure to 1 uM BCES, an abnormally low percentage of cells appeared to be able to replicate their DNA between 3 and 6 days post-exposure (Table 1).

TABLE 1

Labeled nuclei in control and BCES-exposed monolayer cultures of keratinocytes pulse-labeled with [^{3}H]thymidine.

Days in Culture	% Labeled Cells ± S.D.	
	Solvent Control	BCES
4	30 ±4	12 ±3
5	14 ±3	12 ±3
6	32 ±4	22 ±6
7	27 ±3	18 ±4

Cultures were exposed to 1 uM BCES for 1 hour after 1 day of cultivation and pulse labeled for 2 hours with [^{3}H]thymidine on the indicated days of subsequent cultivation. Control cultures were handled similarly except for the exposure to BCES. Labeled cells were counted by autoradiography as described by Vaughan, et al (1976). Data are the percentages of cells with densely labeled nuclei in each type of culture.

DNA synthesis was further studied in unexposed monolayer cultures up to 7 days after plating. Figure 5 shows the specific activity of tritiated DNA at different times after cultures were pulse labeled for 3 hours with [^{3}H]thymidine. Two, four and six days were identified as peak periods of DNA synthesis during the cultivation. Counting of labeled cells by autoradiography confirmed these times of peak labeling (Figure 6) and suggested that the culture underwent three waves of synchronous DNA replication during the 7-day period. It should be emphasized that, as will be discussed below, during the period of 3 to 7 days of cultivation a large percentage of the cells are in the differentiated stage and do not replicate their DNA. In the newborn rat *in situ*, only cells in the basal layer of the cutaneous epidermis in the newborn rat, replicate their DNA and undergo mitosis. In the culture, nearly all of the cells present in the monolayer at Day 1 replicate their DNA during the next 24 hours. This was demonstrated by the observation that nearly all the cells were labeled when the label was continuously present during the period. During the second and third cycle, only 40 and 60 percent of the cells, respectively, became labeled when the tracer was present continuously through the period.

When cultures were exposed to 1 uM BCES for 1 hour at day 1, then allowed to grow in normal medium for 6 additional days and

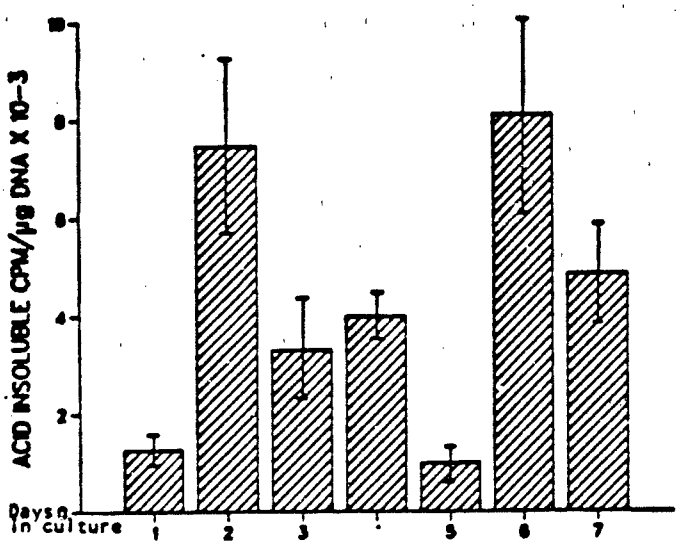


Figure 5. Specific radioactivity of DNA at different times during the cultivation period after exposure for 3 hours to [^{3}H]thymidine. For methodology see Legends to Figures 3 and 4.

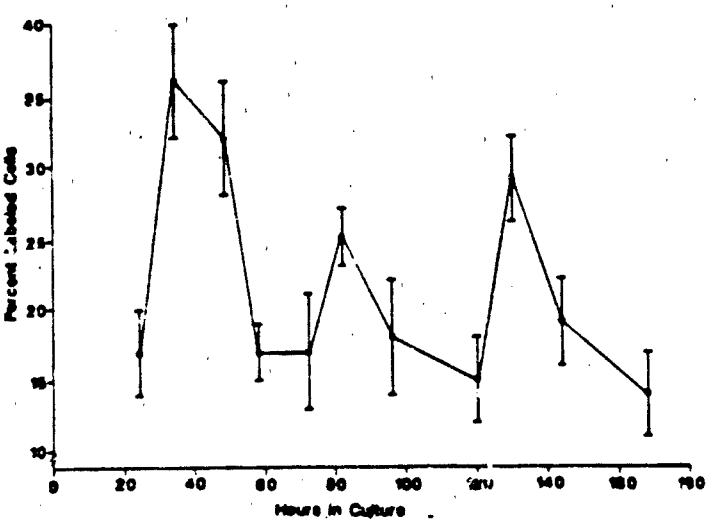


Figure 6. Percentage of labeled cells in the culture at various times after exposure for 3 hours to [^{3}H]thymidine. Autoradiography was carried out as described by Vaughan, et al (1976).

pulse-labeled for 3 hours with [^{3}H]thymidine as had been done with the untreated cultures, the autoradiographic data shown in Figure 7 were obtained. No heavily labeled nuclei were seen until 3 days after exposure and only the last two waves (at 4 and 6 days) of DNA replication were seen. The incorporation of the tracer 24 hours after the exposure to BCES was probably a result of repair synthesis, based upon the fact that the labeled cells had relatively few silver grains over the nuclei. The specific activity of tritiated DNA was 30 percent of normal at 24 hours post-exposure and 18 percent at 48 hours post-exposure. Replication appears to have resumed at 72 hours post-exposure although the percentage of cells undergoing replication of DNA was only 40 percent of normal at this time; thereafter, the percentage of cells replicating DNA in the treated culture was always lower than that in the untreated culture (Figure 7).

In summary, it appears that exposure of a monolayer culture to 0.1 uM BCES for 1 hour is sufficient to cause supercoiled nuclear DNA to relax as a result of the insertion of single strand breaks in

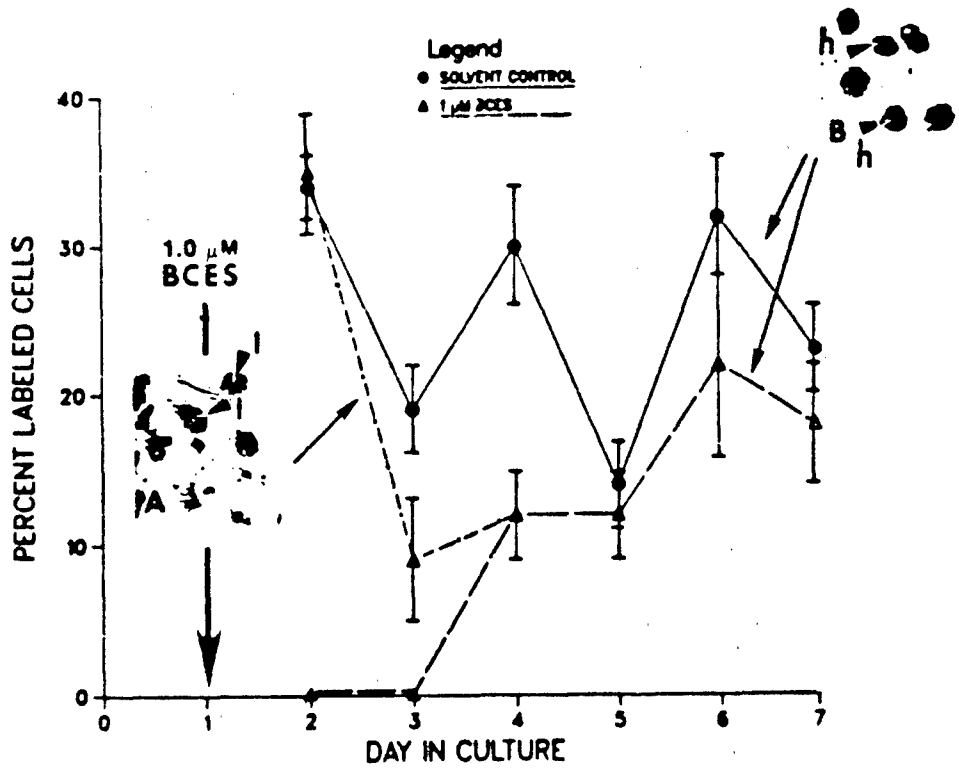


Figure 7. Effect of BCES on the labeling of cells by [^{3}H]thymidine. Autoradiography was carried out according to Vaughan et al (1976). l = lightly labeled nuclei; h = heavily labeled nuclei. Data are given as percentage of heavily labeled nuclei in each type of culture.

the molecule. Although the DNA is repaired and the supercoiling is restored, about 20 percent of the cells are unable to incorporate [^{3}H]thymidine at 24 hours post-exposure. Cells are still able to restore the supercoiling of DNA after an exposure to 1 uM BCES, but only about 80 percent of the cells survive and a substantial fraction of the remaining putative proliferative population is unable to replicate DNA 1 at 6 days post-exposure. Exposure to higher concentrations of BCES results in greater damage and increased numbers of dead cells.

2). Mitosis

In a monolayer culture, the number of cells increases between 1 and 3 days of cultivation (Figure 8). Although the total number of cells does not double in that period, a substantial portion of the population does exhibit mitosis. This was demonstrated using the selective staining procedure reported by Fraser (1982). As mentioned above, it appears that nearly all of the cells present in the monolayer at 24 hours of culture double their DNA during the subsequent 24 hours. If this were true, a doubling of the population should occur shortly thereafter. However, as shown in Figure 8, the population of the control culture goes from 5.2×10^5 at day 1, to 4.3×10^5 at day 2, to 5.6×10^5 at day 3. The failure to observe a doubling of the population is probably a function of the precipitous loss of cells from the monolayer which occurs during this same period. Apparently, many cells in the inoculum attach initially but deteriorate and are lost between days 1 and 3.

As shown in Figure 8, after an exposure to 1 uM BCES for 1 hour, there is no increase in the number of cells until day 5 when a low rate of increase becomes apparent. After an exposure to 5 uM BCES for 1 hour, there is a considerable loss of cells and no increase in cell number is seen during the 7-day experimental period.

In summary, exposure of a monolayer culture to as little as 1 uM BCES for 1 hour inhibits mitosis until day 5 post-exposure. Higher exposures inhibit mitosis even longer. These data are complementary to the previously described data indicating that exposure to 1 mM BCES inhibits DNA replication until day 4. Replication of DNA seems to precede mitosis by about 24 hours.

3). Differentiation

From the results described in subsection 2), it appears that exposure to 1 uM BCES causes a loss of cells predominantly in the germinative population, leaving an abnormally high fraction of differentiated cells in the culture. To substantiate this conclusion, lectin-binding techniques were utilized to measure the relative concentrations of germinative (basal) cells and differentiative (spinous) cells in the culture.

The surfaces of cells in the cutaneous epidermis of the newborn rat exhibit a discrete change in the specificity of lectin binding as the cell leave the basal layer and move into the lowest layer ofspinous

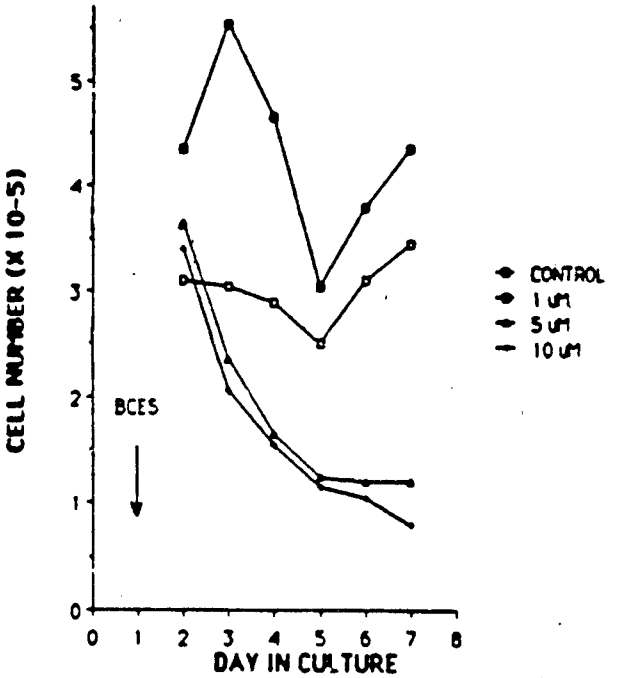


Figure 8. Effect of exposure to BCES on the number of cells in the culture.

cells. The isolectin Griffonia simplicifolia I-B_4 (GS I-B_4) shows preferential binding to basal cells. Ulex europeus Agglutinin I (UEA) preferentially binds to the surfaces of spinous and lower granular cells and is thus a marker of epidermal differentiation. Therefore, the ratio of binding by GS I-B_4 to that by UEA can be used to estimate the ratio of the proliferative to the differentiative cell compartment in the culture.

In vitro, submerged monolayers of keratinocytes derived from the skin of the newborn rat and maintained in a medium low in calcium ion exhibit a characteristic pattern in the ratio of bound UEA to bound GS I-B_4 over a 7-day period of culture when assayed by a quantitative fluorometric assay for lectin binding (Ku and Bernstein, 1986) (Figure 9). The assay involves exposing the culture to GS I-B_4 labeled with fluorescein isothiocyanate (FITC) and UEA labeled with rhodamine, removing the unbound fluorescent lectin probes by washing the culture, eluting the bound lectins using the sugars which bind specifically to the respective lectins (alpha-methyl-D-galactoside for I-B_4 and alpha-L-fucose for UEA), and then determining the amount of each lectin liberated by measuring in a spectrophotofluorometer the fluorescence at a wavelength characteristic for each of the fluorescence labels (FITC excited at 496 nm and emission recorded at 520 nm; rhodamine excited at 556 nm and emission determined at 580 nm). The ratio of UEA to GS I-B_4 is initially low at 1 and 2 days, increases steadily to a maximum at about 3 days and then decreases to a low at 6 to 7 days. This pattern is interpreted to mean that the fraction of differentiated cells increases between 2 and 4 days and decreases thereafter. This interpretation was supported by fluorescence micrographs of cultures at 2, 4 and 6 days stained with FITC-labeled GS I-B_4 and rhodamine-labeled UEA (Figure 10). The percentage of UEA-labeled cells

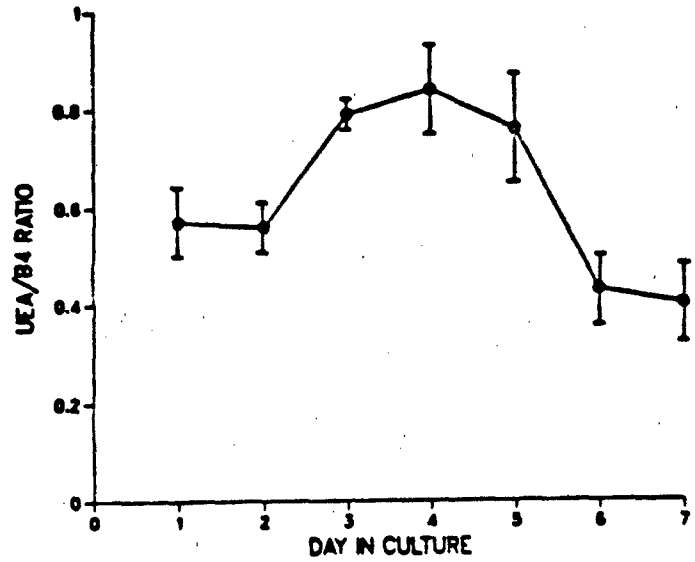


Figure 9. UEA/I-B_4 at various times during the cultivation of the monolayer. For methodology see the TEXT.

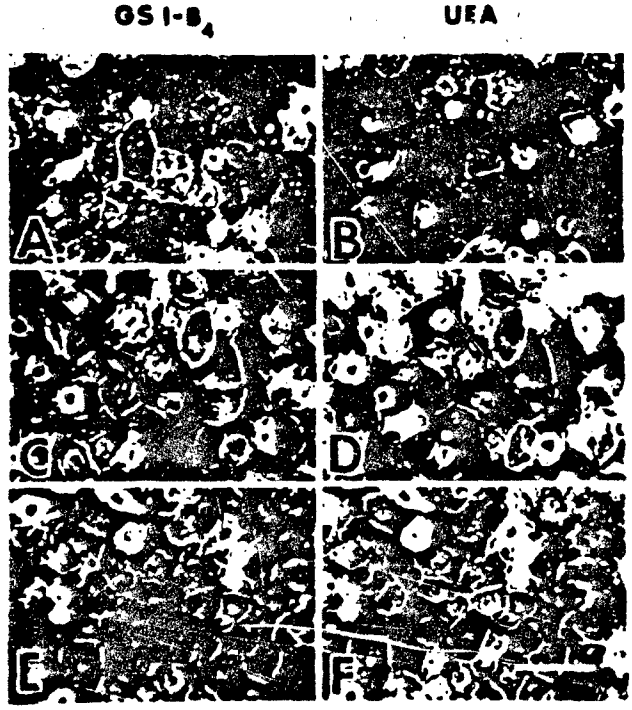


Figure 10. Fluorescence micrographs of cultures exposed to fluorescently tagged UEA and I-B_4 at 2 (A,B), 4 (C,D) or 6 (E,F) days. Staining and microscopic procedures were described by Brown, et al (187). Each pair of micrographs (e.g., A and B) represent the same field in the culture.

was clearly greater at 4 days than at 2 or 6 days. The decrease in the ratio of binding of GS I-B_4 to UEA between 4 and 6 days was a result of the loss of differentiated cells from the culture. The micrograph in Figure 11 shows that the sloughed cells bound primarily UEA and not GS I-B_4.

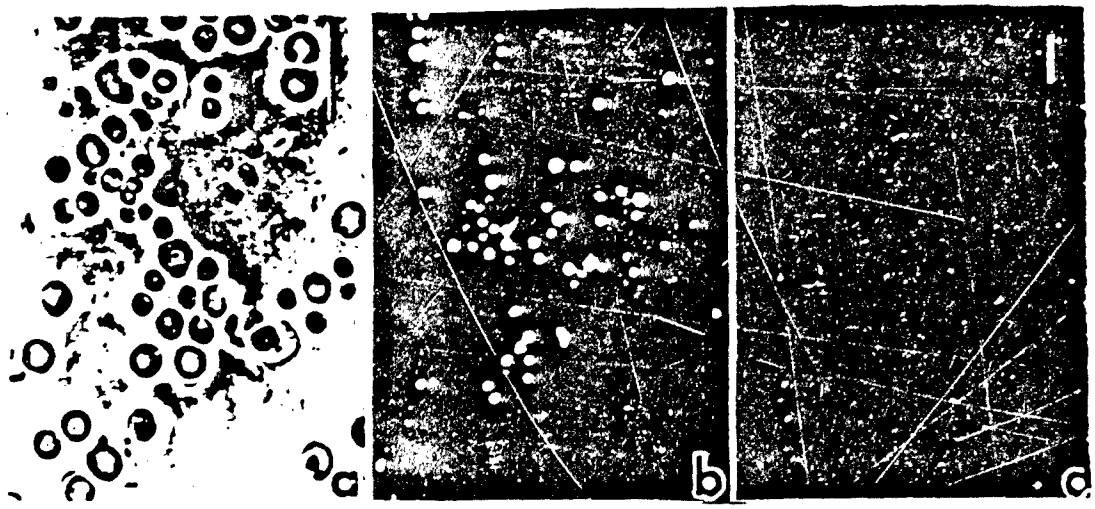


Figure 11. UEA and GS I-B_4 binding to sloughed cells. (a) phase contrast. (b) UEA staining, (c) GS I-B_4 staining. Staining and microscopic procedures were described by Brown, et al (1987).

Figure 12 shows the results of exposing submerged monolayer cultures to 1 uM BCES for 1 hour at 1 day of cultivation followed by 6 days of incubation in growth medium. At the end of each day of cultivation the ratio of binding by the two lectins was determined and is plotted in the figure. The main deviation from the control culture was seen at 5 - 6 days post-exposure, when the ratios of UEA to GS I-B_4 for the exposed cultures were 50 percent higher than those for the controls. At this time, the exposed cultures contained a large number of enlarged cells in contrast to with the control cultures (Figure 13). These enlarged cells were first seen at 3 days post-exposure.

In summary, cultures exposed to 1 uM BCES showed major changes in the binding of lectins to their cell surfaces at 5 - 6 days post-exposure when compared to control cultures of the same age. It appears that there is a decrease in the proliferative population and an increase in the differentiative population at this time. This conclusion is supported by the presence of large numbers of unusually large cells in the exposed cultures at 5 - 6 days post-exposure.

In a complementary study, the effect of BCES on the differentiative changes in keratin peptides was investigated. A monoclonal antibody (2D6) to a 54.5 Kd keratin peptide found in the cutaneous epidermis of the newborn rat was used as a probe in this work.

The 2D6 antibody, conjugated with FITC, stains only the epidermal basal layer of cells in tissue sections of newborn rat skin

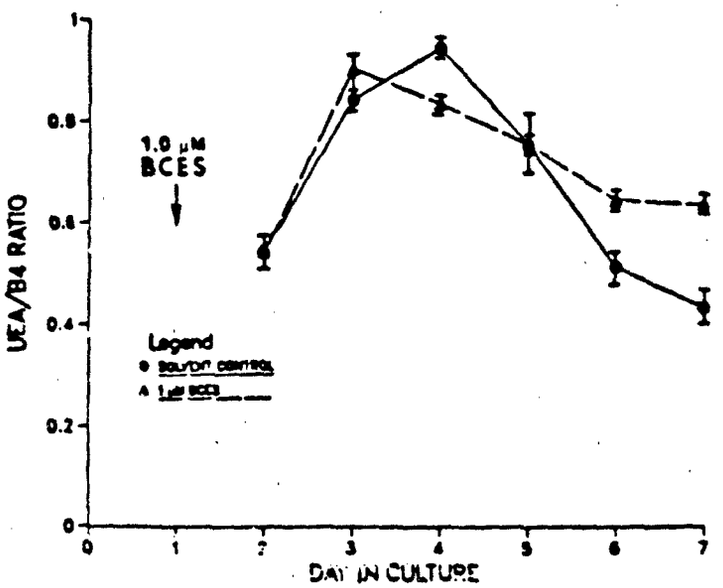


Figure 12. Effect of BCES on the ratio of UEA to GS I-B_4 binding. Cultures were exposed to 1 uM BCES for 1 hour at time indicated by arrow. BCES was removed by washing and cultivation was continued in fresh medium.

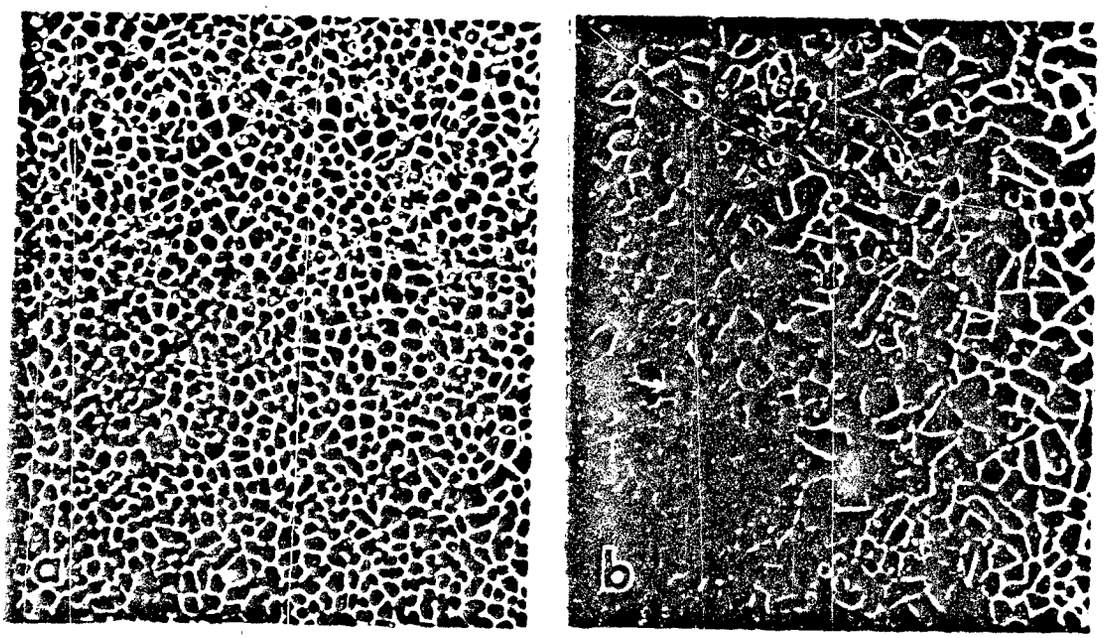


Figure 13. Phase contrast micrograph of normal and BCES-treated monolayer on day 6. (A) normal, (B) treated. Arrows indicate large cells.

prepared in a cryostat. However, after pretreatment of the tissue sections with alcohol, the antibody binds to all viable cells. The peptide can also be isolated from the differentiated cells of the epidermis. This keratin peptide, therefore, is present throughout the viable portion of the epidermis *in situ*. The antibody binds to >95 percent of the cells in the lowest layer of a Percoll density gradient obtained by sedimentation of a mixture of cells derived from trypsinization of newborn rat skin. These cells are used as the inoculum for preparation of monolayer cultures. After 24 hours of cultivation, 2D6 stains about 60 percent of the monolayer. This percentage remains the same through 3 days of cultivation.

When cultures grown for 1 day were exposed to BCES for 1 hour and incubated in fresh growth medium for 24 hours, there appeared to be a dose-responsive increase in the percentage of cells which bound 2D6 (Figure 14). At an exposure to 0.5 uM BCES, a slight decrease, probably insignificant, was observed. At 1 uM, the labeled cells constituted about 115 percent of those in control cultures. At 10 uM, the increase was to about 142 percent. At 20 uM, no increase was seen.

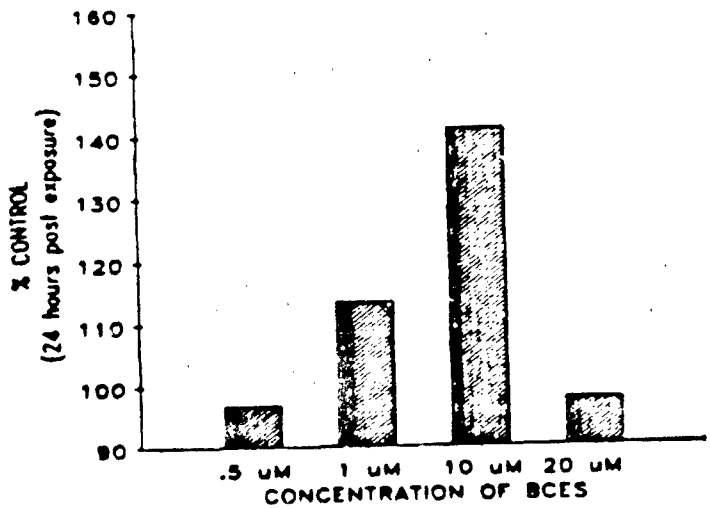


Figure 14. BCES-induced change in 2D6-binding. Binding was evaluated by counting fluorescing cells after exposing the culture to 2D6, washing to remove unbound antibody, and exposing the culture to goat anti-mouse IgG conjugated with fluorescein isothiocyanate.

It appears from these parameters, that 24 hours after an exposure to 1 uM and 10 uM BCES, there is an increase in the percentage of basal (germinative) cells. This conclusion appears inconsistent with other data developed in this project. As noted earlier in this report, there is no replication of DNA during the first 3 days or mitotic activity during the first 4 days afterexposure to 1 uM BCES. There are several possible explanations for this paradoxical observation regarding the keratin polypeptide. First, a large percentage of the cells lost after exposure could consist of differentiated cells which do not bind 2D6. Subsequent analysis would then produce what appears to be an increase in the percentage of cells which bind 2D6. Analysis of 2D6 binding among cells which were lost from the culture as a result of exposure to 1 uM or 10 uM BCES showed no difference between treated and untreated cultures in the percentage of cells which bound 2D6 (Table 2).

TABLE 2

Binding of 2D6 to cells lost from control and treated cultures during the 24 hours of growth post-exposure

	Percentage of 2D6-Positive Cells		
Dose of BCES	Exp. 1	Exp. 2	Exp. 3
Control	10	18	20
1 uM	--	17	18
10 uM	12	18	25

After exposure, sloughed cells were stained and counted (cf. Brown, et al, 1987, for technique) and the percentage of positive cells calculated. The cells were made permeable with Triton X-100, exposed to the antibody, washed to remove unbound antibody, and exposed to goat anti-mouse IgG conjugated with FITC, which binds to the antikeratin. Fluorescing cells were counted microscopically.

There are two additional possible explanations for the effect. Either there is a failure to mask the 2D6 determinant, as occurs normally during differentiation, or BCES has caused cells which had differentiated to synthesize new 2D6 determinant. The former intuitively seems more likely but both alternatives need to be considered.

A third differentiative marker has been studied with respect to BCES. It has been shown that insulin conjugated with FITC binds preferentially in cells of the spinous layer <u>in situ</u> (Feng, et al. 1987). In stratifying cultures of rat keratinocytes, insulin conjugated with FITC can be used to distinguish among four types of keratinocyte. Type 1 appears to represent basal (germina-tive) cells; Type 2, spinous cells; Type 3, late spinous or granular cells, and Type 4, cornified cells (Figure 15).

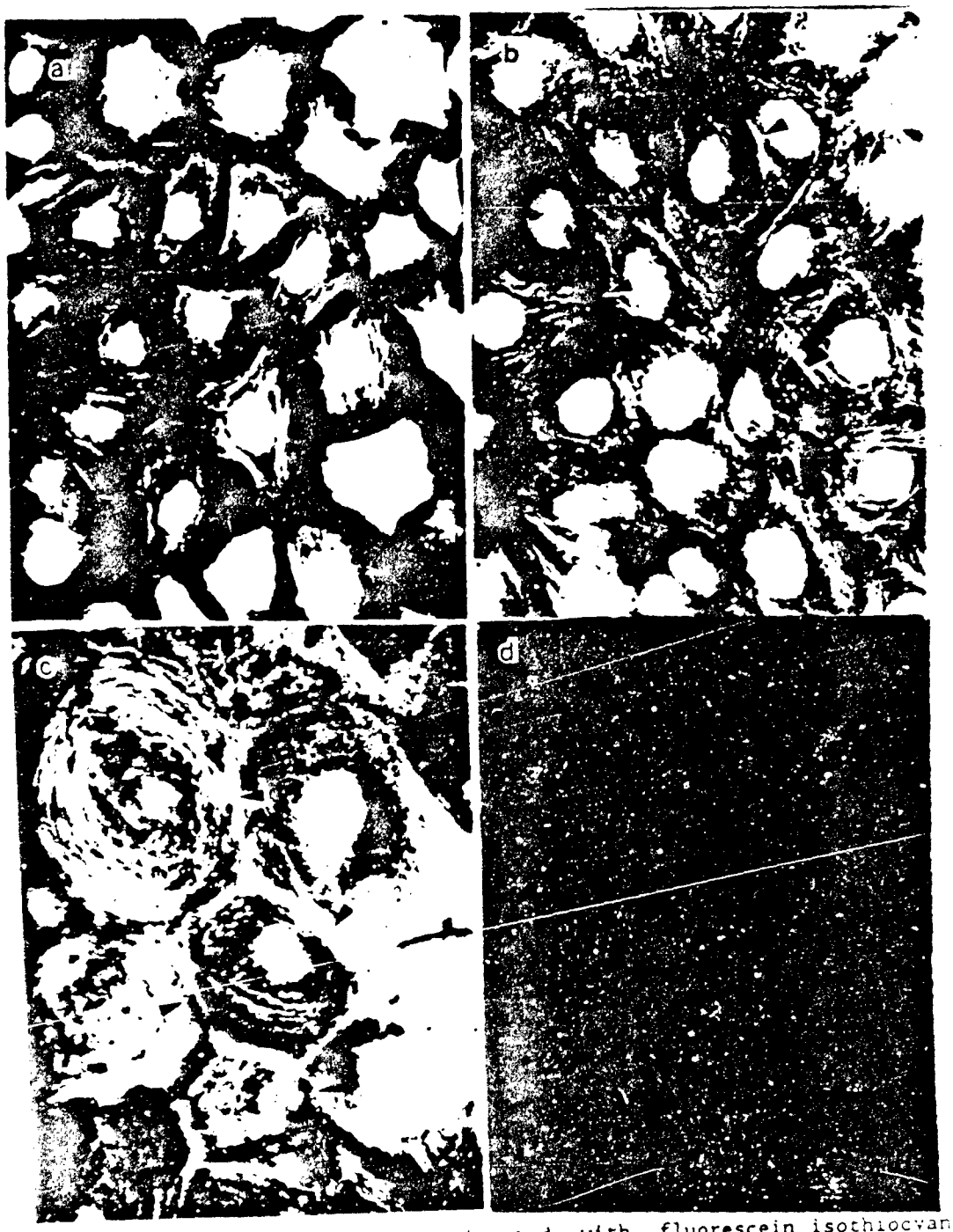


Figure 15. Binding of insulin conjugated with fluorescein isothiocyanate (I-FITC) to cells in a monolayer of normal cells. (a) Type 1 cells (basal cells without I-FITC-decorated cell-to-cell connections). (b) Type 2 cells (spinous cells showing early appearance of I-FITC-decorated (◄) cell-to-cell connections). (c) Type 3 cells (granular cells with mature (◄) I-FITC-decorated cell-to-cell connections) and (d) Type 4 cells (cornified cells with no FITC decoration).

The effect of BCES on differentiation can be probed with conjugated insulin.

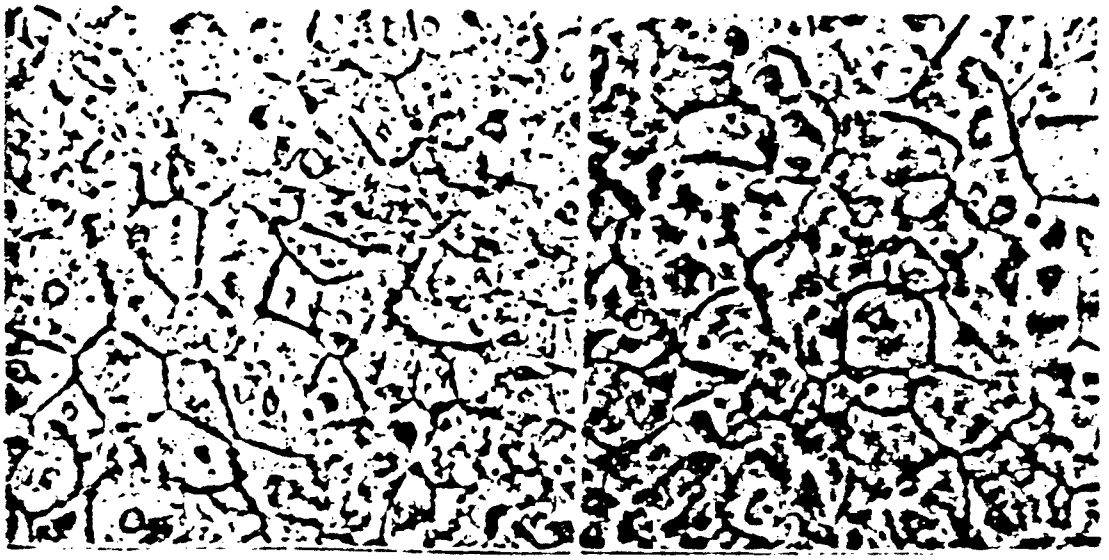
When cultures grown as monolayers in medium with a low concentration of calcium ion were exposed to 5 uM BCES for 1 hour and normal calcium concentration was restored immediately, cells of all four types were seen and no difference was observed between treated and untreated cultures (Figures 16 and 17). However, if the concentration of calcium ion was not raised until 1 day after exposure to BCES, the formation of Type 4 cells was strongly inhibited, although Types 2 and 3 cells were observed. If the increase in the calcium level was delayed for 2 days after exposure to BCES, Types 3 and 4 cells were rarely seen. If the increase was delayed until 3 days, even Type 2 cells did not show the normal binding pattern with insulin. Their normal (Figure 18) cell-to-cell contacts were not extensive. When the calcium level was increased 6 days post-exposure, cells with cytoplasmic filaments were seen but the filaments rarely connected one cell with another as is the case normally (Figure 19).

In summary, when monolayer cultures growing in medium low in calcium were exposed to BCES, the cultures appeared to terminally differentiate normally if the level of calcium in the medium was raised immediately after exposure to BCES. However, the longer the cells remained in the low calcium medium post-exposure, the less complete was the terminal differentiation when the calcium level was finally raised. Apparently, the BCES-mediated damage is exascerbated if cultivation is continued in a low calcium medium but not in a medium with a normal level of calcium. This matter deserves further attention but it is presently difficult to postulate a mechanism to account for the effect.

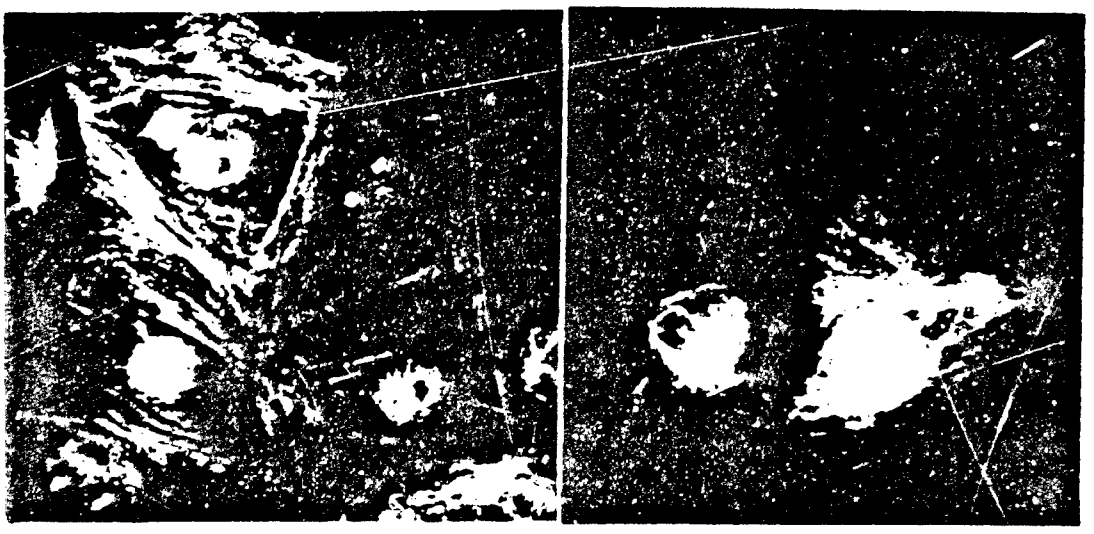
4). Cellular ultrastructure

As noted in subsection 1), reversible macromolecular changes occur when monolayer cultures grown and maintained in a medium of low calcium ion (0.1 mM) are exposed to a concentration of BCES as low as 0.5 uM (Bernstein, et al. 1985). When the exposure to BCES is as high as 5 uM, irreversible changes occur and some cells do not survive the exposure. To ascertain the possible relevance of these macromolecular changes to morphological damage, electron microscopic studies of cultures exposed to BCES in the same dose range were done.

Monolayer cultures of cutaneous keratinocytes from newborn rats were grown from an inoculum of 7.5×10^5 cells per 35 mm dish in a medium of low calcium ion (0.1 mM) for 2 - 3 days. The cultures were then exposed to 0.8 - 0.9 and 4.0 - 4.5 uM BCES for 1 hour. The BCES in methylene chloride was diluted into Eagle's Balanced Salt Solution or phosphate buffered saline for addition to the cultures. The same amounts of the solvents were added to the control cultures as to the BCES-treated cultures. After the exposure, the cultures were rinsed in three changes of phosphate buffered saline and returned to the growth medium (Brown, et al. 1987) (MEM containing 10 or 15% fetal calf serum previously treated with chelex and adjusted to 0.1 mM calcium ion, and antibiotics) for further incubation.



Figures 16 (left) and 17 (right). Phase contrast micrographs of differentiated cultures grown from untreated (Fig. 16) and BCES-treated (5 uM for 1 hour) (Fig. 17) monolayers and immediately transferred into normal calcium medium. No differences were observed between the two photographs taken 4 days after the calcium level was raised.



Figures 18 (left) and 19 (right) Fluorescence micrographs of monolayer cultures exposed to BCES and further cultivated in low calcium medium for 1 (Fig. 18) and 6 (Fig. 19) days, then in normal calcium medium for 4 days and stained with insulin-FITC.

Observations made by phase contrast microscopy indicated no differences in appearance of treated and untreated cultures during the first 3 days post-exposure. On the fourth day, however, treated cultures contained very large cells that had large nuclei with accentuated nuclear boundaries; the cells were not seen in normal cultures (Figure 20). The difference between the lower and higher exposure was in the number of enlarged cells per culture rather than in the size of the cells per se. By 5 to 6 days after exposure, cultures exposed to the higher level of BCES had begun to lose cells and by 7 to 8 days after exposure, most of the cells in these cultures were gone. Cultures treated at the lower level of BCES also experienced major loss of cells. Untreated cultures were still confluent at this time.

At the ultrastructural level, no significant differences were seen between untreated and treated cultures at 1 hour and 6 hours post-exposure. At 6 hours treated and untreated cells exhibited ruffled nuclear membranes and some cells had perinuclear vacuoles (Figures 21 - 23). Some of these vacuoles were located between the ruffles of the nuclear membrane but the membrane itself appeared to be intact (Figure 24). The ruffling was seen in some cells in the treated cultures - but not in control cultures - at 20 hours post-exposure (Figure 25). By 48 hours, the phenomenon was no longer visible in any culture (Figure 26). The ruffling of the nuclear membrane was probably the result of the stress caused partly by the handling of the cultures and partly by the exposure to BCES.

Cells from cultures exposed to both levels of BCES had more vacuoles and many more mitochondria than the untreated cultures. The mitochondria in treated cultures were smaller and showed signs of deterioration as compared with those in untreated cells. At 48 hours after exposure, treated cells were larger than control cells and their cytoplasm contained fewer ribosomes, more tonofilaments, more mitochondria, and various types of granules (Figure 26), including secondary lysosomes, small dark granules that might have been necrotic mitochondria, large dark granules and empty vacuoles that probably contained lipid material (Figure 27). Damage to the tonofilaments was seen only in cells exposed to the higher concentration of BCES only. The tonofilaments in these cell had lost their fibrillar appearance (Figure 28).

Nuclei of some cells from exposed cultures appeared to contain inclusions at 20 hours post-exposure. These inclusions usually contained granular matrix which resembled ribosomes or glycogen granules (Figures 23, 25 and 29). Similar structures were also observed sometimes in the cytoplasm. The cytoplasmic granules were observed less frequently than were the nuclear granules. These cytoplasmic structures resembled the cytoplasmic vacuoles described in cells from human skin exposed to very high doses of BCES (cf. Papirmeister, et al. 1984b). No nuclear inclusions were seen at 48 hours after exposure suggesting that the presence of the structures was transient.

In summary, exposure to low concentrations of BCES, 1/20th of that believed to be fixed in the basal cells of the skin exposed to moderate concentrations of BCES (Papirmeister, et al. 1984a), causes a slight and reversible ruffling of the nuclear membrane and a reversible

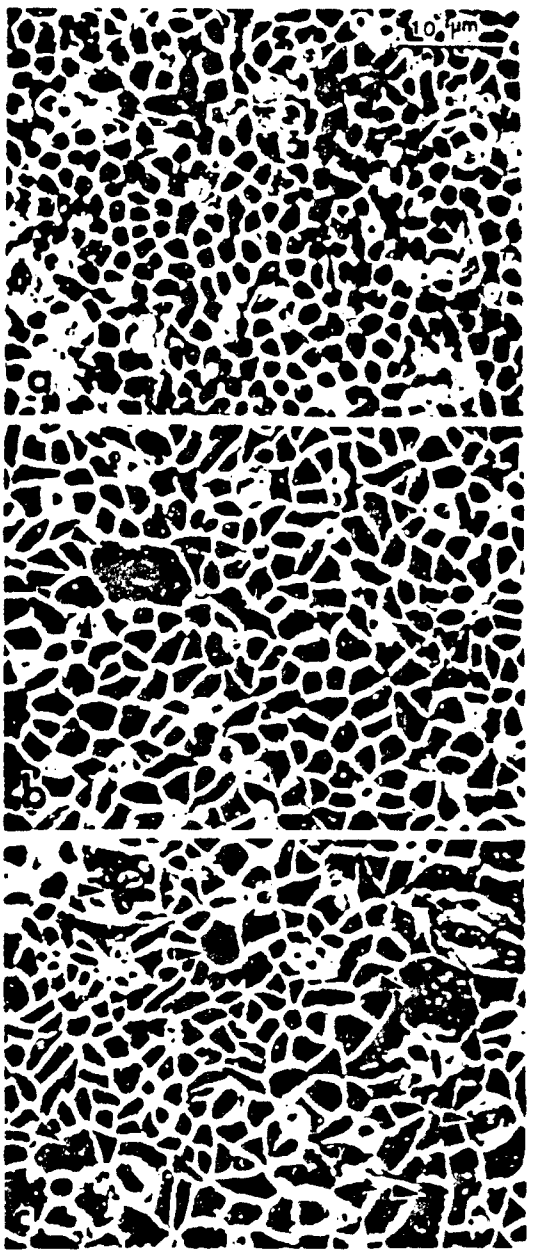


Figure 20. Phase contrast micrographs of low calcium monolayers of rat keratinocytes 4 days after exposure to BCES. At 3 days, low calcium monolayers were exposed for 1 hour to BCES, rinsed and cultivated for 4 additional days in low calcium medium. (a) Untreated culture, 7 days old. (b) Culture exposed to 1 uM BCES. (c) Culture exposed to with 5 uM BCES. (◀) Large abnormal cells. x 200.

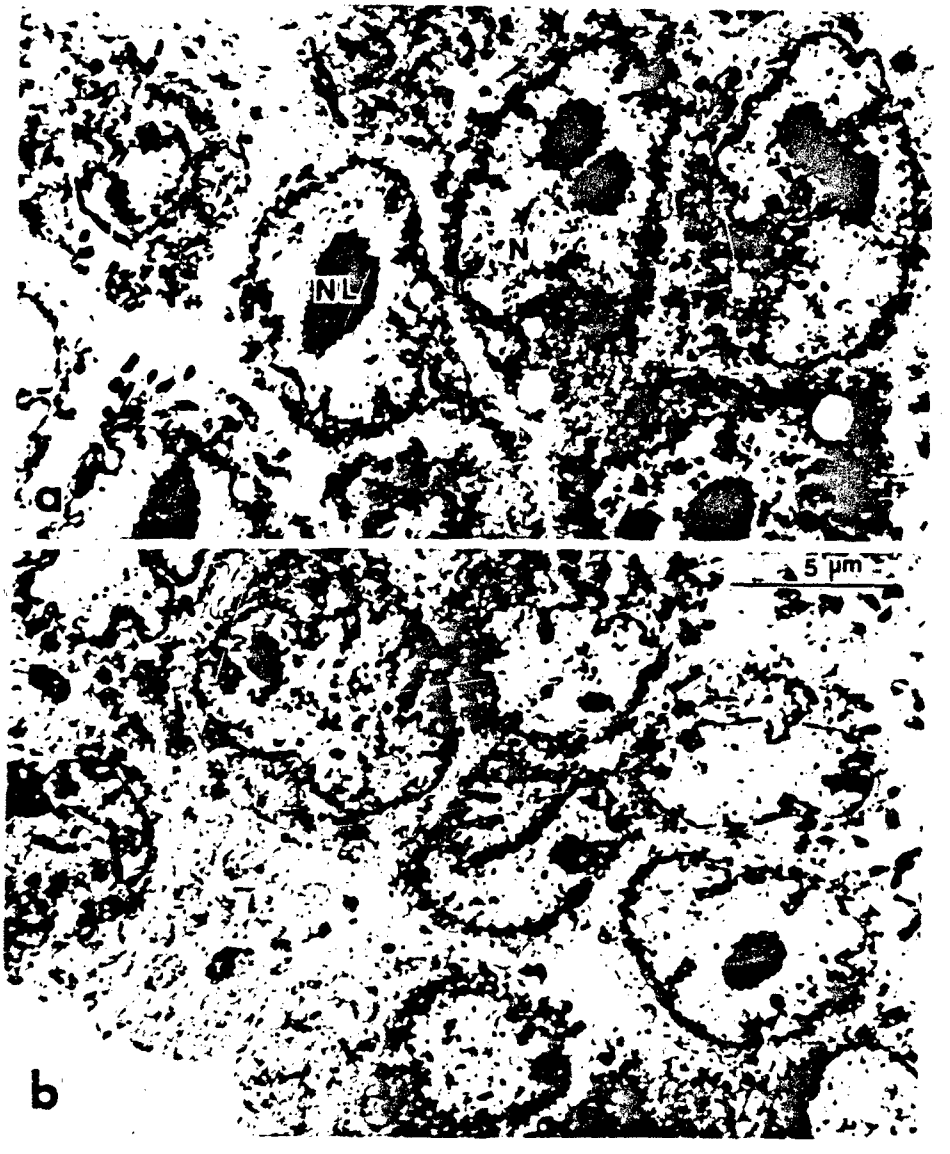


Figure 21. Electron micrograph of untreated and methylene chloride-treated monolayer cultures. Three dayd cultures were first rinsed in 3 changes of Eagle's Balanced Salt Solution (EBSS) (control and solvent control, respectively). (a) Control culture. (b) Culture exposed to methylene chloride (solvent control). Note the moderate ruffling of the nuclear membrane and few cytoplasmic vacuoles. N, nucleus; NL, nucleolus.

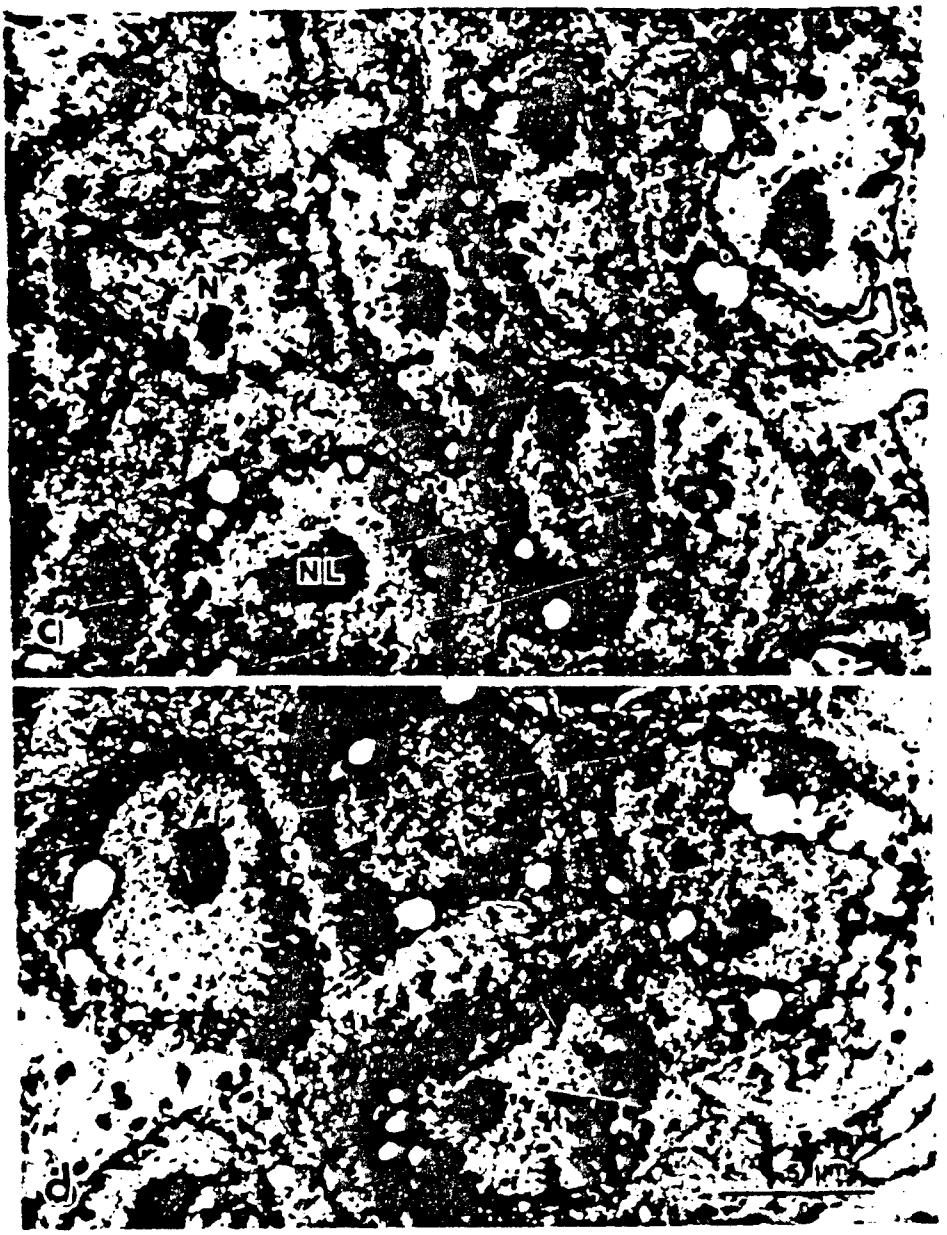


Figure 22. Electron micrographs of monolayer cultures immediately after exposure to 1 and 5 uM BCES. Three-day cultures were exposed to 1 uM BCES (a) or 5 uM BCES (b). Note the ruffled nuclear membrane and the large number of cytoplasmic vacuoles.

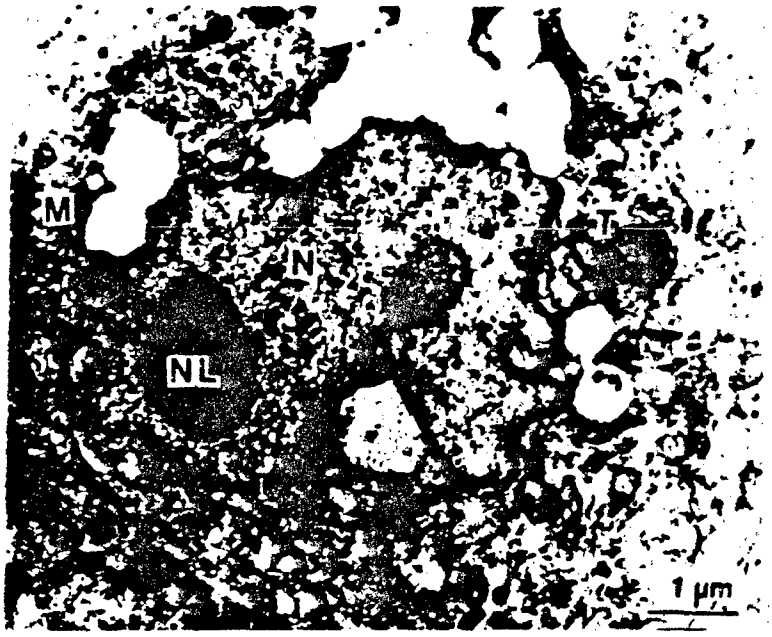


Figure 23. Electron micrograph of monolayer culture 6 hours after exposure to 5 uM BCES. At the end of treatment, cultures were rinsed in 3 changes of EBSS and then incubated for 6 hours in low-calcium medium. Note the vacuoles adjacent to the nuclear membrane and the nuclear inclusions (*). M, mitochondria; T, tonofilaments.

Figure 24. Electron micrograph of a cell as in Figure 23. The nuclear membrane (arrowheads) appears normal. Note "empty spaces" inside the mitochondria. DM, damaged mitochondria; V, vacuole.

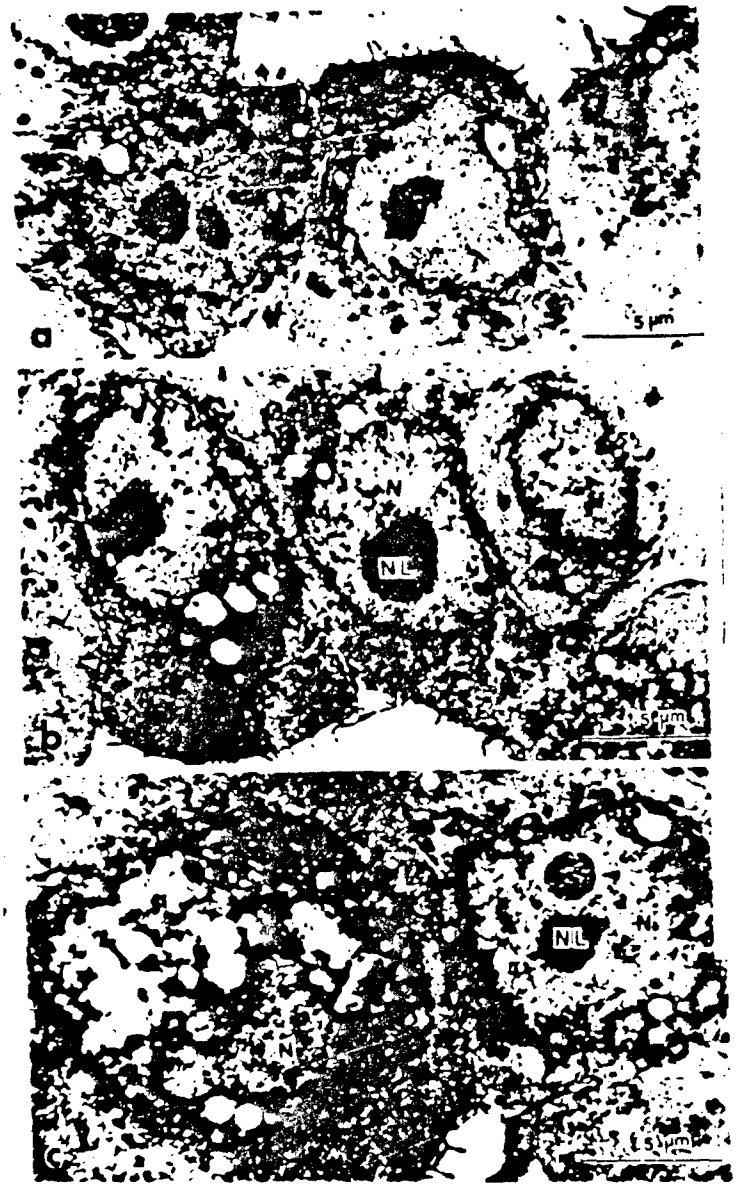


Figure 25. Electron micrographs of control monolayer cultures and cultures 20 hours after exposure to 5 uM BCES. Two-day cultures were first rinsed in 3 changes of PBS, then incubated with PBS, PBS containing methylene chloride (control and solvent control, respectively), or PBS containing BCES for 1 hour. At the end of treatment the cultures were rinsed in 3 changes of PBS and then incubated for 20 hours in low calcium medium. (a) Control culture; (b) culture exposed to 1 uM BCES; (c) culture exposed to 5 uM BCES. The nuclear membrane is not ruffled (compare with Figures 21 and 22), but the treated cells have more cytoplasmic vacuoles than the untreated cells. Note nuclear inclusion (arrowhead).

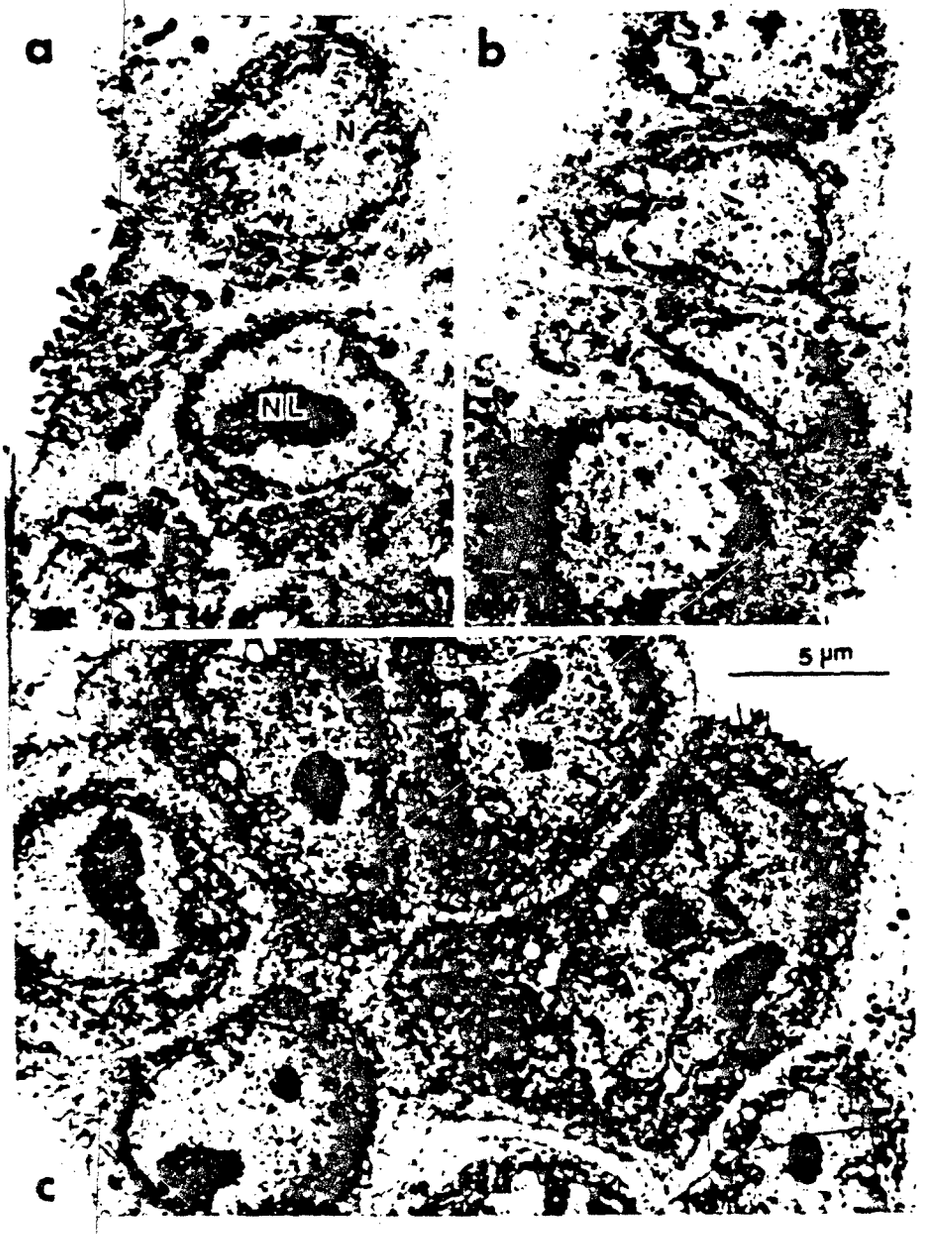


Figure 26. Electron micrographs of control monolayer cultures and cultures 48 hours after exposure to 5 uM BCES. Cultures were treated as described in Figures 21 and 22 and incubated in low calcium medium for 48 hours. (a) Control culture; (b) solvent control culture; (c) culture treated with 5 uM BCES. Cells in control and solvent control cultures are similar to cells from untreated cultures. The cells that were exposed to BCES contain many small mitochondria, granules and tonofilaments.

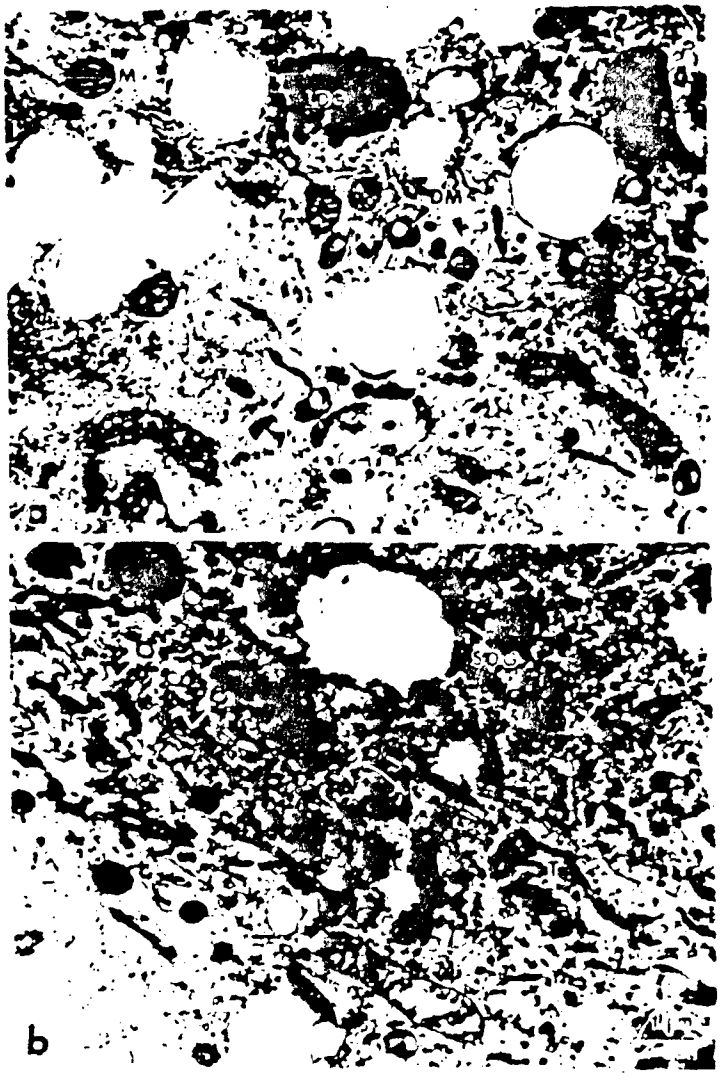


Figure 27. Electron micrographs of 2 cells (a and b) 48 hours after exposure to 1 uM BCES. After exposure, cultures were incubated in low-calcium medium for 48 hours. Some of the mitochondria appear normal (M) while others have deteriorated and appear as small granules (DM). Large dark granules (LDG), small dark granules (SDG), large "empty" vacuoles and small vacuoles (arrowheads) are frequently found in cells from treated cultures.

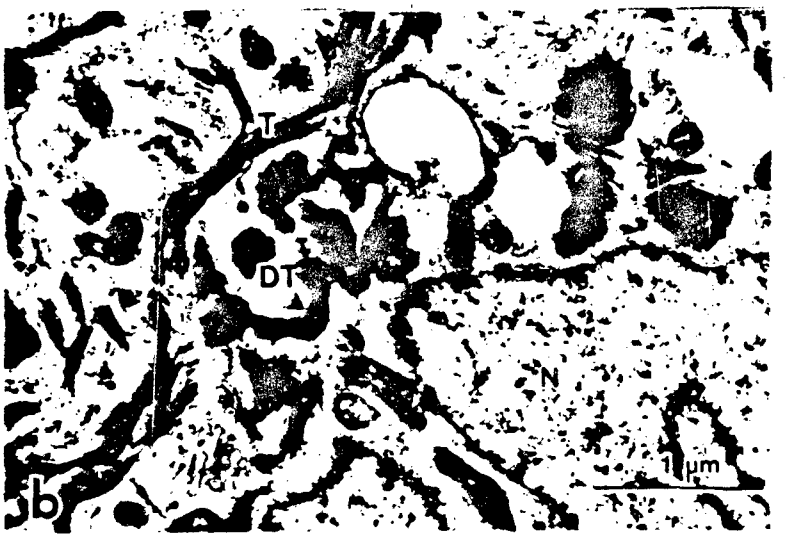


Figure 28. Electron micrograph showing damaged tonofilaments in cells from culture that was exposed to 5 uM BCES and then incubated in low calcium medium for 48 hours. DT, damaged tonofilaments.

Figure 29. Electron micrograph of a cell from a culture treated with 5 uM BCES as described for Figure 25. Note nuclear inclusions (arrowheads) and the large number of mitochondria (M), some of which are deteriorating.

vacuolization of the nucleus and cytoplasm. However, the permanent damage that occurs is expressed at 3 - 4 days post-exposure and includes enlargement of the cells, an increased number of mitochondria and an early loss of cells from the culture. Exposure to about 1 uM BCES is sufficient to cause these effects. The change in the ultrastructure of the tonofilament appears later than the nuclear and mitochondrial abnormalities and requires a higher level of exposure to BCES. From these results, it appears that 0.1 to 1.0 uM is the optimal range in which to study mechanisms of BCES toxicity. Both macromolecular and cellular changes result from this range of exposure. At an exposure of 5 uM, BCES causes generalized cellular damage; it would be difficult to determine primary toxic responses after this exposure.

c. Establishing that lifted cultures, topically exposed to BCES under reproducible conditions of application and penetration of the mustard, exhibit the same abnormalities found in submerged monolayers.

1). Preparation of human lifted cultures

Although the preparation of lifted cultures of human keratinocytes using the techniques reported for primary cutaneous keratinocytes from the rat (Vaughan, et al. 1986; Bernstam, et al. 1986) was previously reported (Bernstein, et al. 1985), the efficiency of the process was not satisfactory for producing the large number of cultures necessary for this project. Furthermore, the biochemical similarity of the cultured tissue and the tissue taken from the human had not been entirely established. During the period covered by this report, several aspects of the procedure for preparation of the human lifted culture were revised in order to make the process more effective, and the biochemical similarity of the culture and the tissue <u>in situ</u> was demonstrated.

Obtaining purified populations of germinative cells from human foreskin has been difficult because of variability in size of the tissue and in yield of cells. Separation of the dermis from the epidermis in adult specimens of skin presents much less of a problem and cultures have been grown from the skin of donors between 32 and 72 years of age. Figure 30 illustrates the gross separation of the dermis from the epidermis from adult skin using the procedure reported for skin from the rat (Vaughan, et al., 1981).

Sedimentation in gradients of Percoll, rather than Ficoll as previously described (Vaughan, et al. 1986), can be employed to obtain populations of human germinative cells essentially free of fibroblasts. This change has been made in the protocol for obtaining epidermal basal cells from the skin of the rat as well. Figure 31 shows the banding pattern obtained in a Percoll gradient using human cells. Cells from the lower band (density: >1.075 g/cc) are washed in MEM and used to seed the culture dish. The yield of cells from one foreskin has been 22 to 45 x 10^4. In spite of high viability indicated by exclusion of trypan blue (85%), not all of the cultures from newborn skin have been successful. About 35% of the foreskins yielded cells that would attach but not proliferate. The yield of germinative cells from adult skin (2 - 4 cm^2) has been 5 - 6 x 10^6,

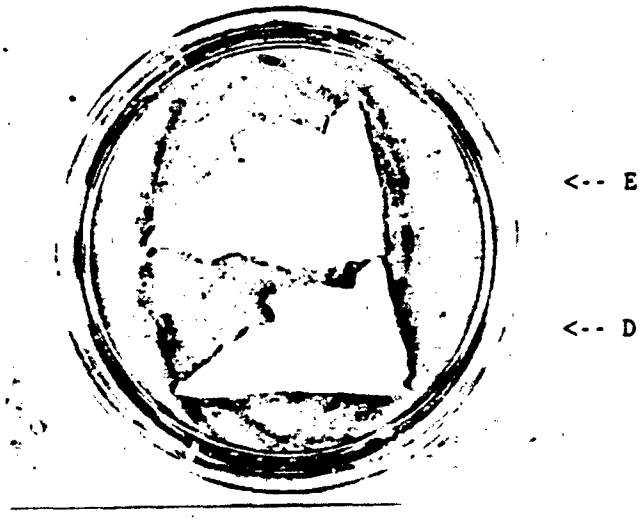


Figure 30. Epidermis (E) separated from the dermis (D) by trypsinization. Adult human skin was used.

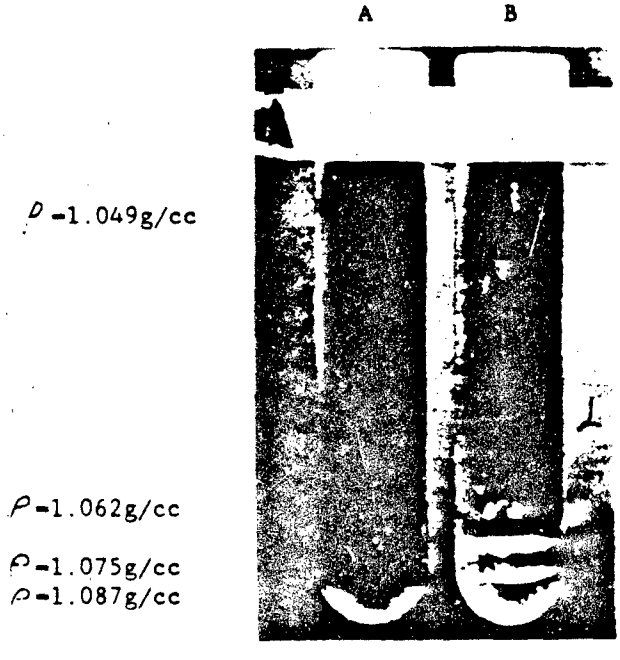


Figure 31. Separation of keratinocytes in a density gradient of Percoll. Cells were from adult human skin. (a) Distribution of beads in the gradient; (b) distribution of cells in the gradient.

and the cultures have nearly always been successful.

For human cultures, cells are plated in 4-well dishes. The cells from one foreskin are usually divided into four portions. Three of the four portions are plated on nylon membranes and put in three of the wells, while the fourth portion is plated directly on the plastic of the well. Since cells growing on a membrane cannot be monitored by phase microscopy, the progress of the cultures on membranes is monitored by observing the growth of the culture on plastic. It has been found that submerged cultures grow comparably on plastic and on the membrane. In the case of adult specimens, 15×10^4 cells are plated per membrane or per well.

When the cultures reach confluence (7 - 10 days for cultures derived from adult skin and 14 days, for those from foreskin), they are lifted to the air-medium interface and growth is allowed to continue for about 3 to 4 weeks. At 21 days, the cultures show 8 - 10 uniform layers of anuclear cells (Figure 32). Complete desmosomes, tonofilament bundles and numerous mitochondria are observed in the viable cell layers (Figure 33). After 28 days of growth at the air-medium interface, about 15 compact cornified layers are observed as are cornified envelopes (Figure 34).

In order to verify the biochemical similarity between the cultures grown at the air-medium interface for three weeks and the tissue <u>in situ</u>, the keratin peptides were isolated by extraction of the entire culture with 0.2M Tris - 8M urea and separated by polyacrylamide gel electrophoresis. The various bands were visualized by staining with Coomassie blue and the 65 - 67 Kd keratins associated with differentiation were identified by immunoblot techniques with the AE2 anti-keratin monoclonal antibody (Sun, et al. 1983) (Figure 35). In order to verify, the location of various biochemical markers of differentiation, cryostat sections of cultures were made and stained with AE2, 3F6 and AKH1 monoclonal antibodies. Monoclonal 3F6 reacts with histidine-rich protein, a major component of keratohyalin (Kim and Bernstein, 1987). AKH1 reacts with filaggrin. (The antibody was kindly provided by Dr. Beverly Dale.) AE2 bound to all cells above the basal layer, and 3F6 and AKH1 bound to the granular cells just below the cornified layer. Figure 36 indicates that each antibody stained the culture at the stage expected from similar experiments done on tissue sections.

In summary, human keratinocytes can be grown on Puropore-nylon membranes to produce stratified, cornified cultures with molecular and morphological markers similar to those seen with epidermis <u>in situ</u>. When adult skin is used as a source of germinative cells, cultures in sufficient quantity for this project can be provided. Cultures from adult skin are better than cultures from newborn foreskin because more precise control of growth can be achieved, the cell yield is greater, more viable cells can be obtained, and plating density is more easily controlled. It has been found that 0.25% trypsin at 4° C for 12 - 14 hours is better for separating the dermis and the epidermis than 0.12% at 4° C or 0.25% trypsin for 60 - 90 min at 37° C.

2). Nucleoid sedimentation assay of damage to DNA in basal cells as a result of topical application of BCES to a lifted culture.

Lifted membrane cultures (21 days) of cutaneous keratinocytes from the rat were exposed topically to dilute solutions of BCES in 70% DMSO. After 2 hours of exposure at 35° C, the cultures were washed with EBSS and then incubated in dilute trypsin-EDTA solution to produce a mixed population of cells (Figure 37). The cell suspension was fractionated on self-forming continuous gradients of 38% Percoll at 30,000 xg for 15 minutes at 4° C. Two major bands were obtained (cf. Figure 31).

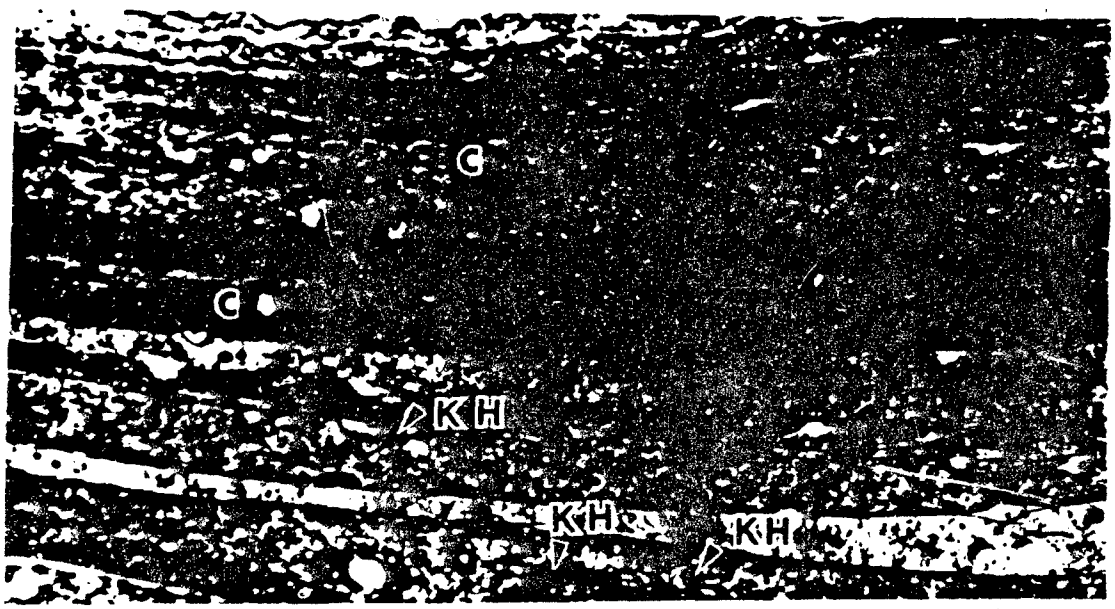


Figure 32. Electron micrograph of a lifted culture of human keratinocytes grown submerged for 2 weeks and lifted for 3 weeks on a nylon membrane. Upper cell layers showing keratohyalin like granules (KH), cornified cells (C). x11,000

Figure 33. Electron micrograph of lifted culture of human keratinocytes grown under the same conditions as in Figure 32. Note the presence of desmosomes (D), tonofiliaments (T), nucleus (NU) and mitochondria (M). x40,000.

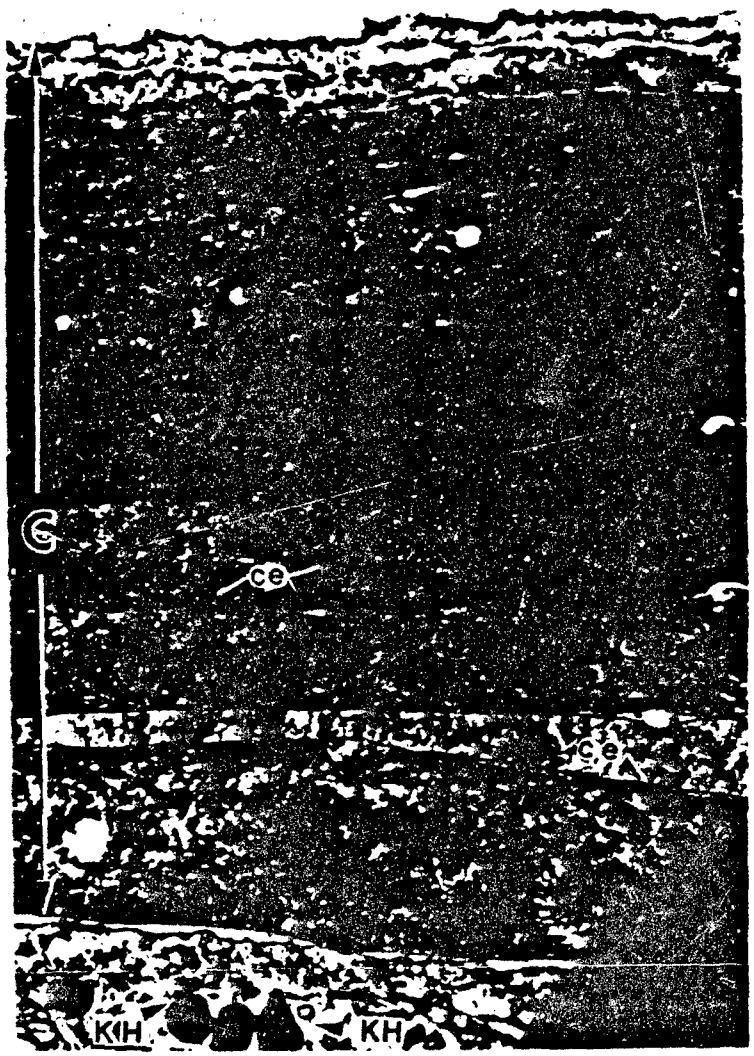


Figure 34. Electron micrograph of lifted culture of human keratinocytes proven submerged for 2 weeks and lifted for 28 days on nylon membranes. Upper cell layers show keratohyalin-like granules (KH) and cornified cells (C) with cornified envelopes (ce).

Figure 35. Immunoblot of keratin (65-67Kd) isolated from stratified cultures by extraction with Tris-8 M urea. Human keratinocytes, grown on nylon membrane for 2 weeks submerged and 3 weeks lifted, were soaked in 0.24 M NH_4Cl for 1 minute at 4°C and separated from the membrane with forceps. Cultures so prepared were homogenized and protein extracts were made in 0.2 M TRIS HCl, pH 8.5, containing 8M urea, 1mM EDTA, 1mM EDGA, 0.1 mM phenylmethanesulfonyl fluoride and 0.1M dithiothreitol. Extracted proteins were separated by electrophoresis in discontinuous gels with 3% stacking and 9% separating gels (Laemmli, 1970) and then were electrophoretically transferred to nitrocellulose. Extracts from human cultures were blocked with 1% bovine serum albumin in PBS for 2 hours, rinsed in PBS and reacted with AE2 antibody overnight. After rinsing, the membrane strips were reacted with second antibody conjugated with peroxidase. Peroxidase activity was detected by staining with 3,3'-diaminobenzidine.

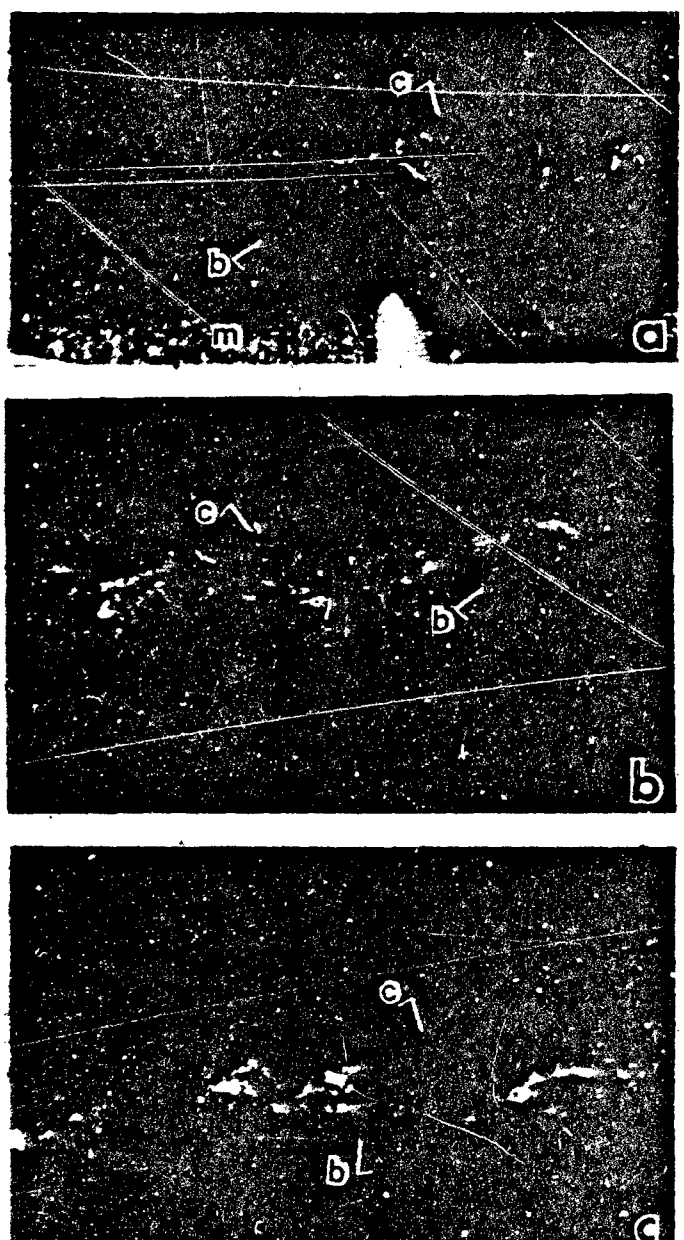


Figure 36. Indirect immunofluorescence staining of frozen sections from a lifted culture with monoclonal antibodies. X 600. (a) 3F6-6 against histidine-rich proteins. (b) AE2 against suprabasal keratin units. (c) AKH1 against filaggrin.

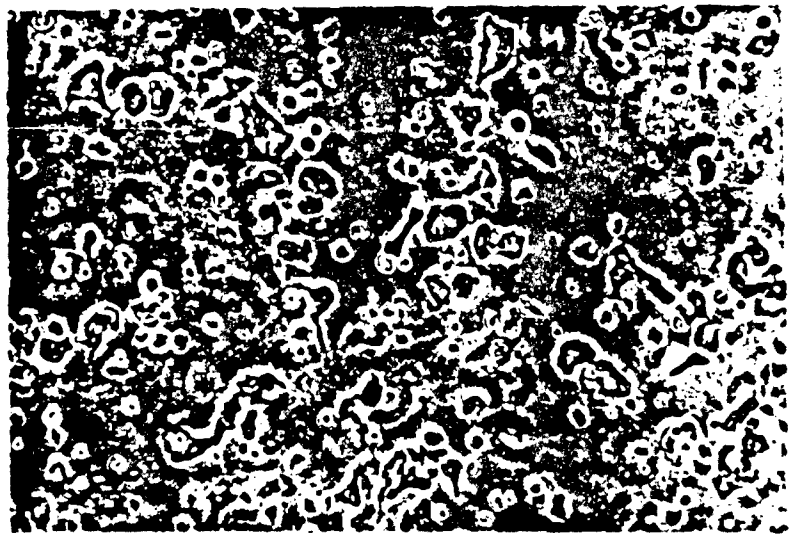


Figure 37. Mixed cell suspension before Percoll sedimentation. Note the heterogeneity in the cell size and shape. Cells comprising the lowest band in the gradient (density = 1.075 g/cc) were harvested, washed and sedimented in the cold (cf. Figure 38).

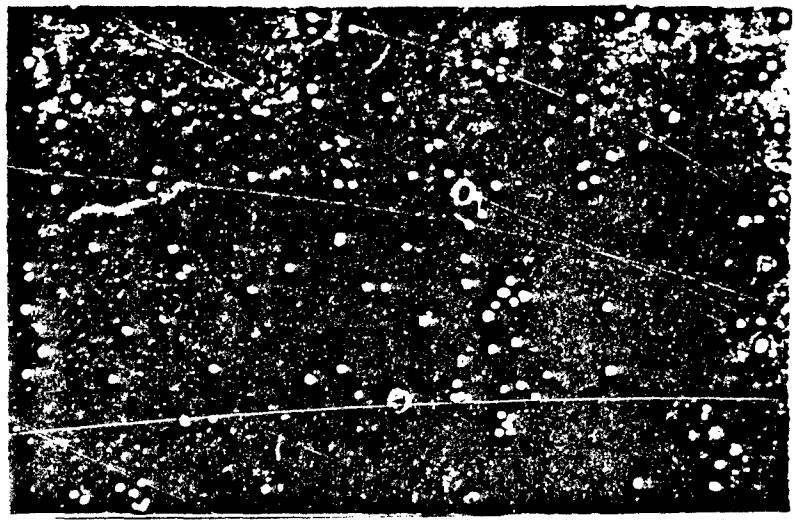


Figure 38. Cells of the lower band in the Percoll gradient. Note the uniformity in cell size and shape.

The cells in the lower band of the gradient are round, compact and have a high nuclear to cytoplasmic ratio. A shown in Figure 38, the population of cells in this band represent a homogeneous population. These are the cells which are used as inocula for growing the cultures and, therefore, represent the proliferative population. As shown in Figure 39, this band contains 95% of the total acid-insoluble radioactivity from a stratified culture that was incubated in the presence of [^{3}H]thymidine for 18 hours prior to harvest.

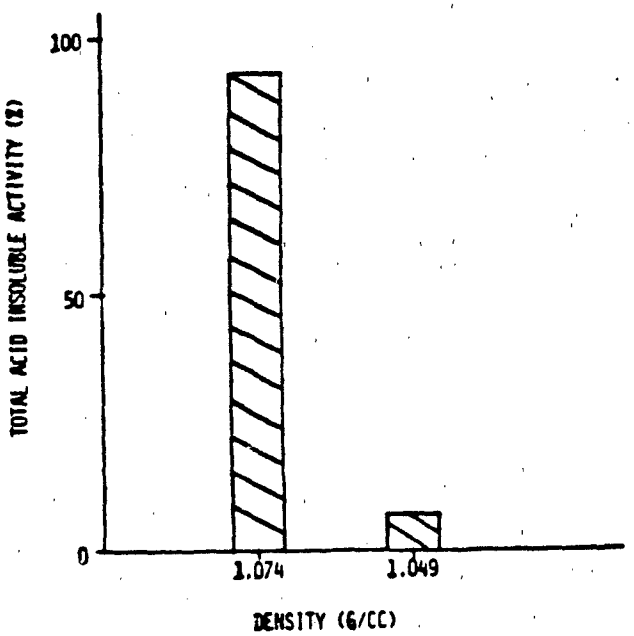


Figure 39. Distribution of [^{3}H]thymidine incorporation in the two major cell bands in a Percoll gradient. Stratified cultures were incubated for 18 hours in the presence of [^{3}H]TdR (0.5 uCi/ml). Cultures were washed and treated with trypsin-EDTA to produce a single cell suspension, which was then fractionated on a Percoll gradient. The two major bands were harvested, precipitated in trichloroacetic acid and solubilized in alkali. A 100 ul aliquot was counted in a liquid scintillation spectrometer.

The nucleoid sedimentation assay was performed on these cells as previously described for cells from a monolayer culture except that

the lysis time was slightly longer (15 to 20 minutes) and the sedimentation was carried out at 10,000 xg for 30 minutes.

A significant and dose-responsive retardation of the sedimentation rate was observed as the dose of topically applied BCES was increased (Figure 40). An exposure of 1 nmol/cm^2 appeared to be ineffective while 200 nmol/cm^2 produced a maximal effect in the assay.

Figure 41 presents data from another experiment, in which BCES had a greater effect than that shown in Figure 40. It is possible that the number of cornified layers was different in the two cases, and one would expect that the greater the number of cornified layers, the lower the amount of BCES reaching the basal layer.

One important conclusion which can be derived from the data in Figure 41, is that, when monitored by the nucleoid sedimentation assay, differentiated cells are less affected by BCES than are basal cells. Exposure to 50 nmol/cm^2 caused a sedimentation value of 58% of control for basal cells but 83% of control for differentiated cells.

Contrary to the situation for submerged monolayers, further incubation for 24 hours did not appear to result in recovery. This apparent paradox needs further attention.

Light micrographs (Figure 42) show no morphological changes in a culture exposed to 10 nmol/cm^2 but considerable damage is present in a culture exposed to 50 nmol/cm^2. The solvent control was no different from the untreated control.

The relationship of the topical dose to the dose applied to a submerged monolayer culture is unknown. However, a higher topical dose is probably required for a particular level of effect, since the mustard must penetrate to the lowest cellular layer through the overlying cell layers in the lifted culture whereas the cells in the monolayer are bathed by the medium containing the toxicant.

In summary, the DNA of the basal cells is more sensitive to BCES applied topically than is the DNA in differentiated cells. The failure to observe DNA repair by this assay in stratified cultures, as was seen in monolayer cultures. Further study of this problem is needed.

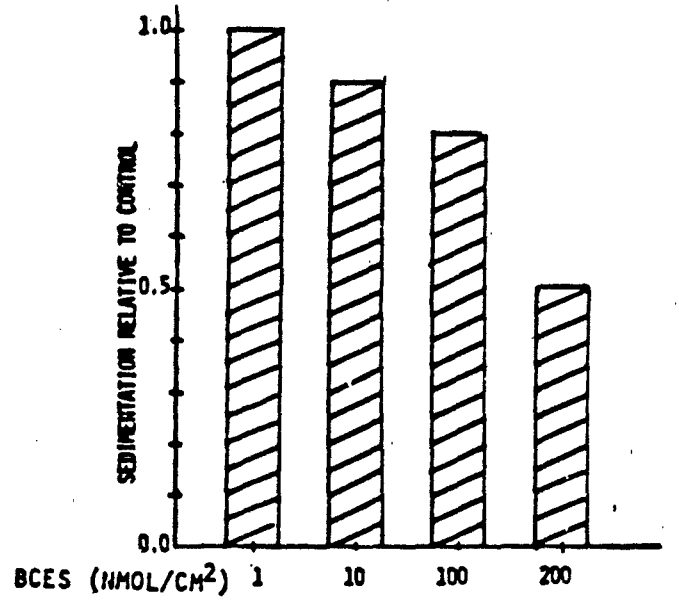


Figure 40. Effect of a topical exposure to BCES on the DNA of basal cells within a lifted stratified culture.

NUCLEOID SEDIMENTATION DISTANCE - % Control AFTER ½ HOUR EXPOSURE TO BCES					
BASAL CELLS					
POST-EXPOSURE TIME (HR)	CONCENTRATION				
	SOLVENT	BCES (nmoles/cm²)			
	70% DMSO	0.01	1	10	50
0.0	100±2.00	97.5±9.19	100±4.72	82.1±9.83	58.1±10.7
24.0	100±3.00	99.5±8.71	100±5.00	84.9±5.40	54.0±2.99
DIFFERENTIATED CELLS					
POST-EXPOSURE TIME (HR)	CONCENTRATION				
	SOLVENT	BCES (nmoles/cm²)			
	70% DMSO	0.01	1	10	50
0.0	100±0.00	99.0±1.41	99.5±0.71	99.6±3.72	83.0±16.5
24.0	100±0.00	-----	97.5±3.54	98.7±6.29	84.0±1.20

Figure 41. Nucleoid sedimentation analysis of DNA in basal and differentiated cells from lifted cultures exposed for 30 minutes to BCES applied topically in 70% dimethylsulfoxide (DMSO). Basal and differentiated cells were separated by sedimentation in a self-forming gradient of 38% Percoll and analyzed separately. For further technical details see legend for Figure 39.

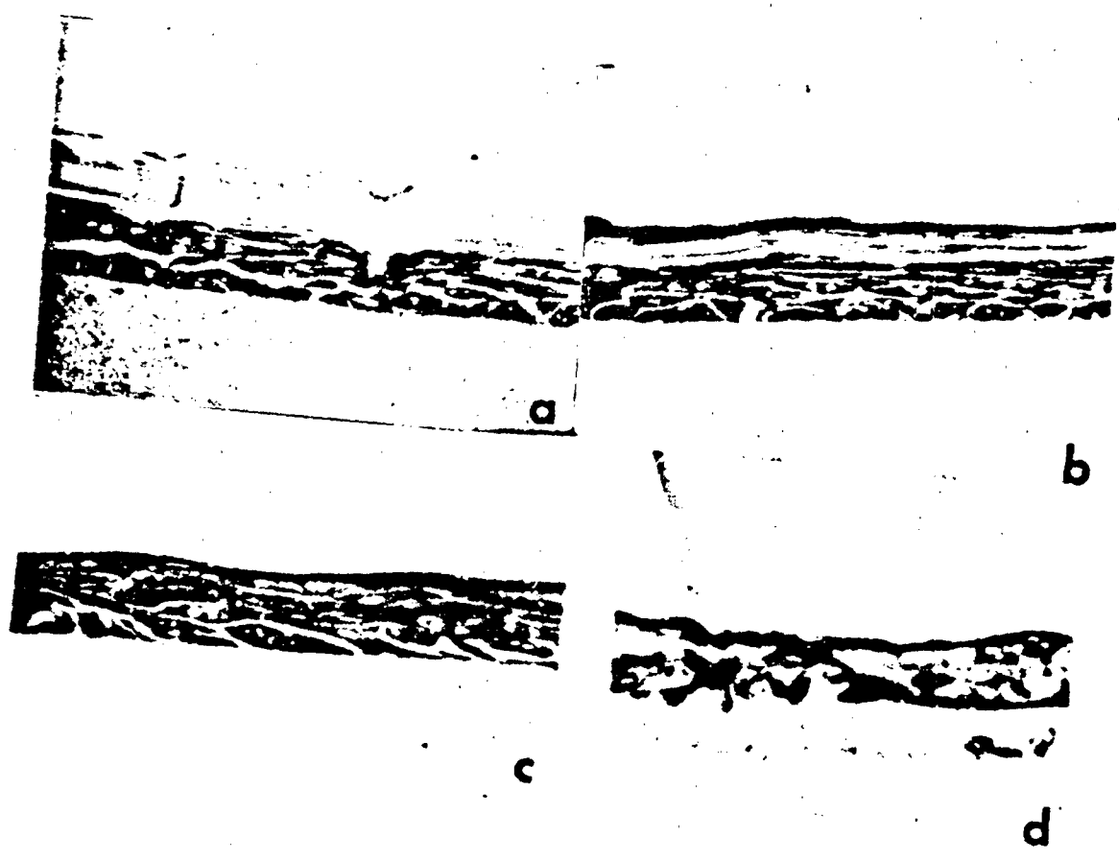


Figure 42. Lifted rat keratinocyte cultures exposed to BCES and control culture. Cells were grown on nylon membranes submerged for 6 days and lifted for 12 days. BCES in 70% DMSO was topically applied for 0.5 hours. In the solvent control, 70% DMSO was applied. At the end of treatment, cultures were incubated for 48 hours in normal medium. (a) Untreated control; (b) solvent control (70% DMSO); (c) 10 nmol/cm^2 BCES; (d) 50 nmol/cm^2 BCES. Horizontal spaces between cell layers in (a) are the result of embedding. x560.

d. Development of an assay to measure fidelity in DNA repair

The use of viruses to investigate DNA repair has become widespread (Defais, et al.,1983). In most such investigations, some modification of the technique of host cell reactivation has been applied. In principle, this experimental technique involves infection of a permissive host with a virus which is non-virulent because of damaged DNA. The method depends on cellular repair processes to reverse the damage and reactivate the virus to virulence. This technique is to be used to determine the efficacy of the host cell's repair system and to evaluate the possible effects of a toxic chemical on the repair process. By inserting a specific lesion into the viral DNA, it should be possible to evaluate the repair system for that particular type of lesion. This technique is being developed for use in evaluating the effect of exposure to BCE on the "error-free" repair capability of exposed keratinocytes.

Specifically, the probe will be a heteroduplex of SV 40 DNA containing two mismatched base pairs which confer temperature sensitivity on the large T antigen genome. This DNA will be used to transfect a culture of human keratinocytes which is semipermissive for SV 40. Repair will then be allowed to occur at a non-permissive temperature so that only the repaired DNA, i.e., the temperature-insensitive genome, will be transcribed and translated to form the large T antigen. T antigen must be made in order for the viral DNA to be replicated. When T antigen is made from temperature sensitive genome, the protein is not stable and viral DNA is not replicated. Repair of this damaged DNA will be monitored by noting the time it takes for the host cells to repair the DNA and the percentage of transfected cells which carry out the repair. The end point assay will be the appearance of new viral DNA (determined by gel electrophoresis) or the appearance of viral plaques.

To date, stocks of wild type SV 40 virus have been prepared and titered by the plaque formation technique, and viral DNA has been isolated and purified according to the Hirt procedure (Hirt, 1967). Transfection of BSC-1 monkey kidney cells, the permissive host, has been achieved using indirect fluorescent antibody detection of T antigen as end point (Lai, 1980). Human cutaneous fibroblasts in culture have also been successfully transfected by the calcium co-precipitation technique (Graham and van der Eb, 1973). The percentages of transfected cells in both cases are much less than was the case with infection of the monkey kidney cells. Human keratinocytes have not yet been transfected, although it has been done in another laboratory (Banks-Schlegal and Howley, 1983).

G. Publications from this project in 1986:

1. Vaughan, F.L., Gray, R.H. and Bernstein, I.A. (1986) Growth and Differentiation of Primary Rat Keratinocytes on Synthetic Membranes. In Vitro, Cell. Develop. Biol., 22, 141-149.

2. Bernstam, L., Vaughan, F.L. and Bernstein, I.A. (1986) Keratinocytes Grown at Air-Liquid Interface. In Vitro, Cell. Develop. Biol., 22, 695-705.

3. Ribeiro, P.L., Mitra, R.S. and Bernstein, I.A. (1986) A Sensitive Assay for the Determination of Low Levels of DNA Damage in Primary Cultures of Rat Epidermal Keratinocytes. The Toxicologist, 6, 240.

4 Ku, W.W. and Bernstein, I.A. (1986) Lectin Binding as a Probe of Irritant-induced Changes in Keratinocyte Proliferation and Differentiation In Vitro. The Toxicologist, 6, 239.

5. Zaman, S., Scavarelli, R., Vaughan, F.L. and Bernstein, I.A. (1986) Macromolecular Metabolism of a Differentiated Keratinocyte Culture System Following Exposure to Sulfur Mustard. The Toxicologist, 6, 277.

H. Plans for Year 2

1. Complete dose response curves for damage to DNA, inhibition of mitosis, alteration in differentiation and change in ultrastructure resulting from exposure of monolayered cultures to BCES; prepare manuscripts for publication of these results.

2. Complete dose response curves for damage to DNA in lifted cultures exposed to BCES topically and demonstrate autoradiographically that BCES penetrates through the tissue in order to affect the basal cells.

3. Continue the study of repair of BCES-mediated lesions in the DNA of lifted cultures after topical application of the mustard.

4. Investigate the nature of the lesion in the DNA (and the mechanism of its repair).

5. Investigate the effect of BCES on the fidelity of repair in order to evaluate the hypothesis that errors in the repair process are responsible for the failure of exposed cells to completely recover after exposure to mustard.

6. Study the mechanism of BCES-mediated abnormalities in differentiation.

7. Continue the effort to establish that BCES causes the same effects in lifted cultures as it does in monolayer cultures.

I. Recommendations

The low level exposures utilized in this study are appropriate for elucidating the primary target site for BCES injury. The monolayer and stratified cultures should continue to be useful in this study.

All results to date could be explained by subtle changes in the DNA induced by exposure to BCES followed by error-prone repair resulting from the effect of BCES on the repair system. This hypothesis should be tested by determining whether BCES causes the DNA repair system to operate at suboptimal fidelity.

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